Use of 4-aminophenol and *p*-phenylenediamine derivatives for the detection of bacterial hydrolyases

Michael Ford

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For David and Helen

Abstract

A range of novel substrates for the detection of bacterial hydrolyases have been examined in both liquid and solid media. The potential inhibitory effect of these substrates on both Gram-positive and Gram-negative organisms has been evaluated. In addition a range of alternative substrates possessing an appropriate chemical configuration have also been evaluated as substrates in this thesis. It has been demonstrated that substrates based on 4-aminophenol, particularly the dichloro derivative produce intensely coloured reaction products upon hydrolysis and subsequent coupling with 1-naphthol. A derivative of 1-naphthol, 3,5-dihydroxy-2-naphthoic acid, was shown to react favourably with a number of released core compounds, producing a wide variety of coloured complexes. In comparison with *o*-nitrophenyl-β-D-galactoside (ONPG), the novel substrates for the detection of β-galactosidase activity showed wide differences in K_m and V_{max} values. Overall these substrates performed well in liquid media, especially the dichloro derivatives for detection of a range of bacterial hydrolyases. The substrates have also been evaluated in solid media and have been shown to produce intensely coloured reaction products. The fact that these substrates offer themselves to the production of novel dual substrate systems indicates their potential diagnostic applications. Use of L-alanyl-DEPPD in combination with 1-naphthyl-β-D-galactoside has been

shown to be of diagnostic potential for the detection of β-galactosidase activity in Gram-negative organisms. In addition, a combination of substrates, L-prolyl-4-amino-2,6-dichlorophenol and 1-naphthyl-β-D-glucoside has also been shown to have potential for the production of a medium specifically for the isolation of *Serratia sp.* This dual substrate system also has applications for the production of media for the specific isolation and detection of numerous clinically important pathogens e.g *Enterococcus sp, C.perfringens* and *Yersinia enterocolitica.*

ABBREVIATIONS

4-amino-GAL	4-aminophenyl-β-D-galactoside
4-amino-AGAL	4-aminophenyl-α-D-galactoside
4-amino-XYL	4-aminophenyl-β-D-xyloside
4-amino-GUR	4-aminophenyl-β-D-glucuronide
4-amino-GLU	4-aminophenyl-β-D-glucoside
4-amino-AFUC	4-aminophenyl-α-D-fucoside
4-amino-BFUC	4-aminophenyl-β-D-fucoside
AFUC	α-fucoside
4-MeU	4-methylumbelliferone
4-amino-AGLU	4-aminophenyl-α-D-glucoside
4-amino-NAG	4-aminophenyl-N-acetyl-β-D-glucosamine
4-APP	4-aminophenyl-phosphate
4-APP-TEA	4-aminophenyl-phosphate-tetraethyl-ammonium salt
4-amino-PC	4-aminophenyl-phosphorylcholine
4-ACP	4-aminophenyl-acetamidophenol (Paracetamol)
7-AMC	7-amido-4-methylcoumarin
β-NAP	β-naphthylamine
β-DCAP	β-alanyl-4-amino-2,6-dichlorophenol
BFUC	β-fucoside
BHI	Brain Heart Infusion broth

CHE	Cyclohexenoesculetin
CIN	Cefsulodin Irgasan Novobiocin agar
DEPPD	Diethyl-*p*-phenylenediamine
DMPPD	Dimethyl-*p*-phenylenediamine
DCAP	4-amino-2,6-dichlorophenol
DMSO	Dimethyl sulfoxide
DCAP-GAL	4-amino-2,6-dichlorophenyl-β-D-galactoside
DCAP-GUR	4-amino-2,6-dichlorophenyl-β-D-glucuronide
DCAP-Phos	4-amino-2,6-dichlorophenyl-phosphate
DCAP-4-ACP	4-amino-2,6-dichloro-acetamidophenol
DCAPGLU	4-amino-2,6-dichlorophenyl-β-D-glucoside
GAL	β-galactosidase
GUD	β-glucuronidase
K_m	The Michaelis constant
K_{cat}	Turnover number for an enzyme reaction
L-ala	L-alanine
L-ala-4AP	L-alanyl-4-aminophenol
L-ala-DCAP	L-alanyl-4-amino-2,6-dichlorophenol
L-ala-DEPPD	L-alanyl-diethyl-*p*-phenylenediamine
L-ala-PNA	L-alanyl-*p*-nitroanilide
L-pyra-DCAP	L-pyroglutamyl-4-amino-2,6-dichlorophenol
L-pyra-PNA	L-pyroglutamyl-*p*-nitroanilide
L-leu-DCAP	L-leucyl-4-amino-2,6-dichlorophenol

L-leu-PNA	L-leucyl-*p*-nitroanilide
MUG	4-methylumbeliferyl-β-D-glucuronide
NAG	N-acetyl-β-D-glucosaminide
o-NP	*o*-nitrophenol
ONPG	*o*-nitrophenyl-β-D-galactopyranoside
ONPAGAL	*o*-nitrophenyl-β-D-galactoside
ONPXYL	*o*-nitrophenyl-β-D-xyloside
ONPGUR	o-nitrophenyl-β-D-glucuronide
ONPGLU	*o*-nitrophenyl-β-D-glucoside
ONPAFUC	*o*-nitrophenyl-α-D-fucoside
ONPBFUC	*o*-nitrophenyl-β-D-fucoside
ONP-Phos	*o*-nitrophenyl-phosphate
ONPA	*o*-nitrophenyl-acetate
p-NA	*p*-nitroaniline
PNPAGLU	*p*-nitrophenyl-α-D-glucoside
PNPNAG	*p*-nitrophenyl-N-acetyl-β-D-glucosaminide
PNPPC	*p*-nitrophenyl-phosphorylcholine
TMPPD	Tetramethyl-*p*-phenylenediamine
V_{max}	The maximum velocity of an enzyme reaction
V_o	Absorbance change per minute
VRE	Vancomycin resistant enterococci
X	Indoxyl

Glossary

Absorption spectrum: A graph of the amount of light a substance absorbs, plotted as a function of wavelength.

Acyl group: a compound derived from a carbonic acid by replacing the hydroxyl group with a halogen (X), usually --Cl; general formula is O R--C—X.

Aglycone: Non-sugar portion of a glycoside.

Aminophenols: phenols substituted in any position by an amino group.

Aminopeptidase: an enzyme, which removes the N terminal amino acid from a protein or peptide.

Anthraquinones: compounds composed primarily of a triple benzene ring, these compounds have a planar structure. Such compounds are dyes and are found in plants such as senna (*Cassia senna*).

β-galactosidase: A sugar-splitting enzyme that catalyses the hydrolysis of lactose into D-glucose and D-galactose. Also known as lactase.

β-lactamase: An enzyme produced by some bacteria, which causes the breakdown of the active form of some penicillin antibiotics, rendering them ineffective. It causes the hydrolysis of a beta-lactam (as penicillin to penicilloic acid); found in most strains of staphylococci, bacteria that are

naturally resistant to penicillin. Synonyms are penicillinase, and cephalosporinase.

Carboxylic acid: a compound containing the group: CO_2H.

Chelating agents: organic chemicals that form two or more coordination bonds with a central metal ion. In microbiology this metal is usually Iron (III). Heterocyclic rings are formed with the central metal atom as part of the ring. Some biological systems form metal chelates, e.g., the iron-binding porphyrin group of haemoglobin. They are used chemically to remove ions from solutions, medicinally against micro organisms, to treat metal poisoning, and in chemotherapy protocols.

Chemiluminescence: Light emitted as a reaction proceeds. In microbiology has been used to detect bacterial ATP (using firefly luciferase) for the detection of urinary tract infections.

Complex: A species made up of a metal ion centre bonded to a number of ligand species.

Compound: a term used generally to indicate a definite combination of elements into a more complex structure (a molecule) but it is also applied to systems with non-stoichiometric proportions of elements.

Delocalization: electron systems in which bonding electrons are not localised between two atoms as for a single bond but are spread (delocalized) over the whole group, e.g. pi-bond electrons, in particular the delocalised pi-electrons associated with aromatic molecules.

Derivative: a compound that can be imagined to arise from a parent compound by replacement of one atom with another atom or group of atoms. Used extensively in organic chemistry to assist in identifying compounds.

Electron withdrawing: An electron-withdrawing group is one which through either mesomeric or inductive mechanisms brings electron density towards itself and away from other areas

Enterobacteriaceae: A large family of Gram-negative bacilli that inhabit the large intestine of mammals. Commonest is *Escherichia coli*, most are harmless commensals.

Excited state: the state of an atom, molecule or group when it has absorbed energy and become excited to a higher energy state as compared to the normal ground state. The excited state may be electronic, vibrational, rotational, etc.

Free rotation: free rotation about a bond means that the energy barrier that must be surmounted to rotate is much less; hence practically all molecules possess enough energy to rotate about a single bond. Double bonds are very fixed and hence rotation about these is fairly impossible, and certainly not 'free'.

Functional group: a functional group is part of a molecule with a characteristic reaction or property. Hence molecules with the same functional groups can be expected to have similarities in their properties.

Glycoside: a compound formed by the reaction of a sugar with a non-sugar molecule called the aglycone.

Glycosidase: a general and imprecise term for an enzyme that breaks up and degrades complex sugar subunits of a polysaccharide e.g. starch or sucrose into simple ones, such as glucose or fructose.

Ground State: the lowest energy configuration of a system.

Hydrogen bonding: hydrogen bonding occurs when a hydrogen atom is attached to an electronegative atom (e.g. N,O,F). The bond between the two is clearly therefore polarised, and another atom with a lone pair can form a bond to this positively charged hydrogen atom. When this occurs between two separate molecules, this is called intermolecular hydrogen bonding.

Indophenols: any one of a series of artificial blue dyestuffs, resembling indigo in appearance, and obtained by the action of phenol on certain nitrogenous derivatives of quinone. Simple indophenol has not yet been isolated.

Kinetics: the study of the rate of reactions.

Hydrolysis: the addition of the elements of water to a substance, often with the partition of the substance into two parts, such as in the hydrolysis of an ester to an acid and an alcohol.

Molecule: the smallest particle of matter that can exist in a free state. In the case of ionic substances, such as sodium chloride, the molecule is considered as a pair of ions, NaCl.

Nadi reaction: formation of indophenol blue by the action of an oxidizing enzyme (cytochrome oxidase) present in bacterial cells and tissues when they are treated with a solution of 1-naphthol and dimethyl-*p*-phenylenediamine.

Oxidation: a chemical process in which the proportion of electronegative substituents in a compound is increased, or the charge is made more positive, or the oxidation number is increased.

Oxidizing agent: is a substance that gives oxygen to another substrance or removes hydrogen from it. Oxidation is the removal of electrons from a substance.

Phenol: hydrocarbon derivative containing an OH group bound to an aromatic pairing.

Polar bond: covalent bond in which there is an unsymmetrical distribution of electron density.

Reducing agent: the substance that reduces another substance and is oxidized.

Reduction: chemical processes in which the proportion of more electronegative substituents is decreased, or the charge is made more negative, or the oxidation number is lowered.

Spectrophotometer: an instrument that measures the degree of absorption (or emission) of electromagnetic radiation by a substance. The measuring system generally includes a photomultiplier.

t-Boc: N-tert-butoxycarbonyl, a chemical preparation of compound often an amino acid which is used to block on end of an amino acid so that another amino acid residue can be added. Used in the preparation of substrates for aminopeptidases for example.

Umbelliferones: 7-hydroxycoumarins. Fluorescent compounds present in many plants and used in sunscreen preparations. Derivatised with carbohydrates to produce highly sensitive fluorescent substrates.

Wursters blue: Blue compound formed by N,N,N,N tetramethyl-*p*-phenylenediamine upon the loss of an electron.

Chapter One

Introduction

Background

The isolation and subsequent identification of a microorganism is still central to the accurate diagnosis of infection, particularly for human pathogens (Langlet *et al.,* 1999). While isolation and laboratory culture has been advanced with the use of a variety of solid media (Cowan, 1974), cultural properties alone are often too variable to provide more than a tentative identification. Whilst several techniques have increased the speed of detection of bacterial pathogens direct from clinical material, most notably by PCR e.g. Lantz *et al.*, (2000) these techniques are extremely time consuming and labour intensive, particularly when used on complex samples (Millar *et al.*, 2000). Thus most routine diagnostic microbiology departments will continue to rely on traditional culture and subsequent identification techniques until such time as molecular techniques are both practical and cost-effective.

Tests for the accurate identification of bacterial pathogens have traditionally relied on the relatively slow detection of carbohydrate fermentation products (Hugh and Leifson, 1953). This situation was transformed by the development of tests for the detection of specific bacterial enzymes (Le Minor and Ben Hamida, 1962). For the first time biochemical tests could be employed which, when used in combination with morphological and

cultural properties, allowed accurate identification and differentiation of numerous bacterial species (Lanyi, 1987).

The quest for new biochemical tests, especially rapid tests, has progressed at a rapid pace, with new substrates (Bainbridge *et al.*, 1991, James *et al.*, 2000b) and novel applications (Kang *et al.*, 1998, and Wiggins *et al.*, 2000) being developed continually.

While many enzyme detection systems utilise natural substrates, enzymes often have a broad specificity, which can be exploited in the design of novel substrates (Bascomb, 1987). Some enzymes, such as urease, are highly specific and fail to attack even closely related substrates; others such as the glycosidases, peptidases and phosphatases show a broader activity (James, 1994). Characteristic enzyme specificity, combined with the ability to catalyse reactions at very low substrate concentrations, has allowed their use for both identification and taxonomic purposes (Miles *et al.*, 1992).

The requirement for more sensitive test methods has led to the development of synthetic substrates for the detection of enzyme activities (Dealler, 1993). In contrast to the traditional use of pH to detect the products of fermentation through a number of metabolic pathways (MacFaddin, 1980), synthetic substrates require only the presence of a single enzyme and involve no further metabolic transformations. Such substrates offer the advantages of

being highly reactive and sufficiently sensitive, with increased specificity, for the rapid detection of a range of enzyme activities in microorganisms (James, 1994) compared to traditional pH detection.

Fluorogenic synthetic substrates.

Synthetic substrates can be placed into three categories: (i) chromogenic, (ii) fluorogenic, and (iii) those requiring the addition of reagents for visualisation (Dealler, 1993). The latter includes substrates of naphthol, phenolphthalein, and depending upon the assay, β-naphthylamide (β-NAP), (Miller and MacKinnon, 1974). Fluorogenic labels include 4-methylumbelliferone (4-MeU), 7-amino-4-methylcoumarin (7-AMC), fluorescein, and resorufin (Fig 1.1).

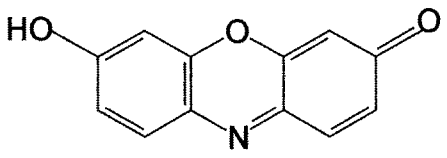


Figure 1.1. Chemical structure of resorufin (Ali-Vehmas *et al.*, 1991). The compound is highly fluorescent (bright pink).

Fluorescence is a highly sensitive method for detecting enzymatic activity, and a wide range of substrates are commercially available, based primarily on 4-MeU (Hartman, 1989). This label is applied most often for the

detection of glycosidase activity in Gram-negative species (Kampfer, 1991) and used most commonly for detection of the both β-D-galactosidase (GAL) and β-D-glucuronidase (GUD). The latter enzyme catalyses the hydrolysis of β-D-glucopyranosiduronic acids into their corresponding aglycons and D-glucuronic acid (Manafi, 1996). The fluorescent substrate 4-methylumbelliferyl-β-D-glucuronide (MUG) is highly sensitive for the detection of GUD, and has been used in a variety of solid and liquid media (Fig 1.2) for the detection of *E.coli,* (Gauthier, 1991) since the majority of strains have been shown to produce GUD (Kilian and Bülow, 1979). The substrate is broken down by GUD to release 4-MeU (Manafi, 1996), which produces blue fluorescence under U.V light (365 nm).

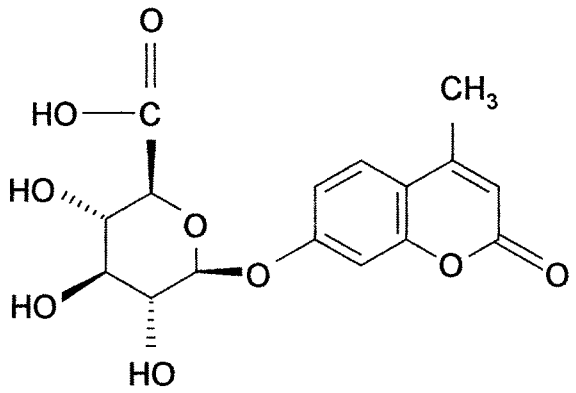


Figure 1.2. Structure of 4-methylumbelliferyl-β-D-glucuronide (MUG).

The substrate, MUG is widely used in the food and water microbiology industries for rapidly confirming the presence of *E.coli* e.g. Sartory and

Watkins (1999). The water industry in particular, with its requirement for the detection of *E.coli,* coliform and *enterococci,* seeks to confirm the presence of these organisms as rapidly as possible (Anon, 1994). The Colilert™ and Enterolert™ systems utilise MUG and 4-methylumbelliferone-β-D-glucoside respectively to achieve this aim. The former was evalutated by Fricker *et al.,* (1997) who indicated the system was a suitable alternative to the current labour-intensive membrane filtration techniques. The use of specific substrates in this area is not without problems however, since environmentally damaged or oxidatively stressed organisms may fail to express the target enzyme (Clark *et al.,* 1991, Martins *et al.,* 1993).

Several other fluorescent substrates have been applied for the detection of various bacterial enzymes, although those based on MUG remain the most widely used. Fujiwara (1978) and several other workers have described the use of 7-AMC for the detection of bacterial aminopeptidase activity. Although fluorescein (Bascomb, 1987), and resorufin (Eggertson, 1999) are also available, these substrates are expensive, limiting their widespread diagnostic use.

While highly sensitive for enzyme detection in liquid media, the problems of rapid diffusion of the label through agar, and high substrate cost compared with chromogenic substrate labels has limited the use of fluorogenic

substrates to certain niche areas of diagnostic microbiology. Doleans (1994) reported the lack of sensitivity of solid media containing fluorogenic substrates when compared to the corresponding chromogenic label. These substrates also require the use of a UV light source for product detection, or complex instrumentation for quantitative analysis (Manafi, 1996). As a result, these requirements have suppressed the widespread utilisation of fluorogenic substrates in diagnostic microbiology.

Chromogenic synthetic substrates

Despite the broad array of different types of substrates and methods of visualisation, perhaps the most widely used are those substrates that release a chromogen upon hydrolysis (Dealler, 1993, James *et al.*, 2000b). Chromogenic labels include ortho- and para-nitrophenols (*o*-NP, *p*-NP), *p*-nitroanilide (*p*-NA), and indoxyl derivatives (Fig 1.3).

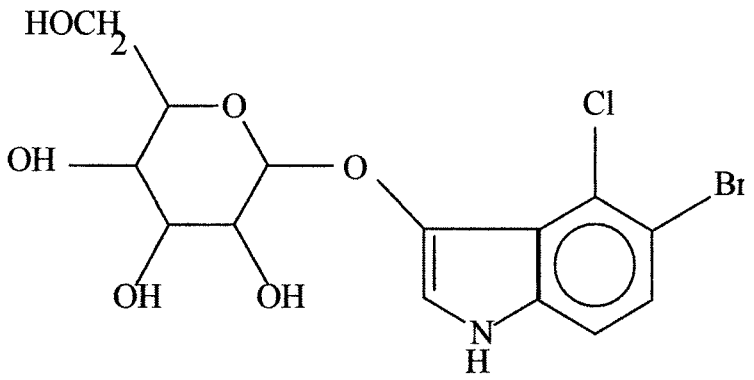


Figure 1.3. Structural formula for 5-bromo-4-chloro-3-indolyl-α-D-galactoside (X-α-GAL).

Indoxyl compounds are now widely used in numerous areas of microbiology, particularly where visualisation of reaction products are required on solid media. Perry *et al.*, (1999), combined the above compound with a recently developed chromogen, cyclohexeneoesculetin-β-D-galactoside (CHE-gal) for the novel detection of *Salmonella* spp. from stool

samples. Although such substrates are widely used in both research and routine microbiology, disadvantages exist, which confine their use to specific situations. The high cost of synthesis, particularly for the highly-coloured halogenated derivatives, has so far limited widespread diagnostic use. Of the other common chromogens, those based on either *o*-NP or *p*-NA are more widely utilised (Bascomb, 1987). However, substrates of *p*-NA are difficult to prepare, due to the electron-withdrawing effect of the nitro group (Hojo *et al.*, 2000). More importantly, use of *p*-NA and *o*-NP substrates is confined to liquid media since the released chromogen diffuses through agar-based media (Manafi, 1996). Substrates of β-NAP release the carcinogenic β-napthylamine (Connolly and White, 1969) and are therefore of limited use due to the potential hazards involved.

The "Nadi" reaction

This reaction was first described by (Erlich, 1885) for the detection of the enzyme indophenol oxidase in tissue. This enzyme, inophenol oxidase catalyses the oxidative coupling of 1-naphthol and dimethyl-*p*-phenylenediamine (DMPPD), hence the "na" and "di" giving the reaction its name. This results in the formation of a deep blue dye - indophenol blue (Figure 1.4). Erlich's work was continued by several authors for the demonstration of indophenol oxidase, (later named cytochrome oxidase) activity in several tissue types (Pearse, 1968). For example, Keilin and Hartree (1937) showed that cytochrome oxidase would catalyse the oxidation of reduced cytochrome c, and produce indophenol blue by the "Nadi" reaction in the presence of atmospheric oxygen.

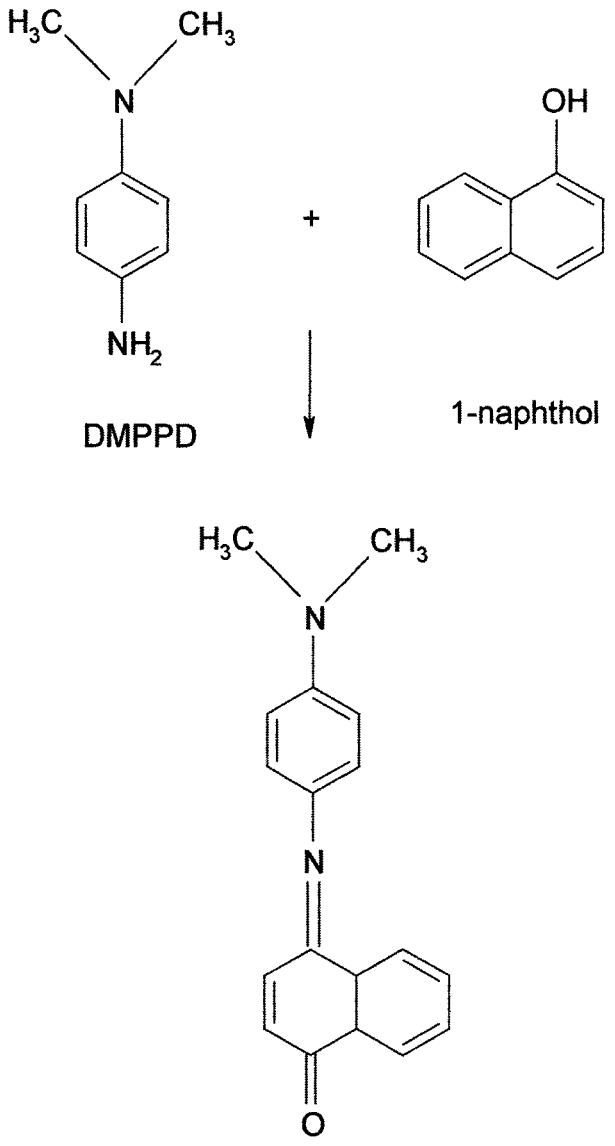


Figure 1.4. Structure of Indophenol blue formed by coupling of DMPPD and 1-naphthol, the "Nadi" reaction.

Indophenol dyes were first described in the German scientific literature of the late nineteenth century by Rohmann and Spitzer (1895) and, following this, by Schultze (1909). Only a few such dyes are now commercially available. Dyes of this class are derivable by oxidative coupling of a phenolic or naphtholic component, with an aminophenol, aromatic 1-4

diamine, or a derivative such as DMPPD. This latter compound has been used in diagnostic microbiology for the differentiation of members of the enterobacteriaceae from *Pseudomonas* spp., the latter possessing substantially greater activity of the enzyme cytochrome oxidase (Gordon and McLeod, 1928). However, the highly chromogenic tetramethyl-*p*-phenylenediamine (TMPPD: Fig 1.5) is more commonly used for this diagnostic test (Kovacs, 1956). Despite this, DMPPD has several advantages as it is more stable than TMPPD (Leclere and Beerens, 1962) and it is more amenable to derivatisation due to the presence of a reactive *p*-amino group.

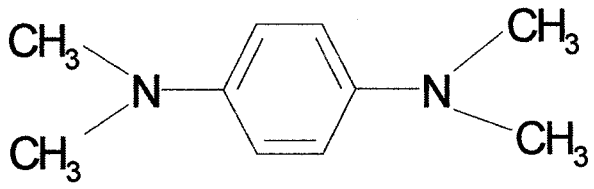


Figure 1.5. Structure of N,N,N,N-tetramethyl-*p*-phenylenediamine (TMPPD).

DMPPD can be linked to an amino acid e.g. L-alanine, thereby creating a substrate for L-alanyl aminopeptidase. Derivatisation with other amino acids offers the potential for detection of a wide variety of aminopeptidases. Possession of the enzyme L-alanyl-aminopeptidase could thus be demonstrated by use of L-alanyl-DMPPD in conjunction with 1-naphthol.

Colour would not develop unless the specific alanyl aminopeptidase acted upon the substrate, shown in fig 1.6, and allowed free *p*-phenylenediamine to undergo coupling. Thus, a deep blue colour would be produced by most Gram-negative species, which possess this enzyme. Such Gram-negative organisms, which are classically oxidase-negative, would rapidly produce a coloured reaction product by virtue of the small amount of enzyme required for the liberation of free *p*-phenylenediamine for coupling.

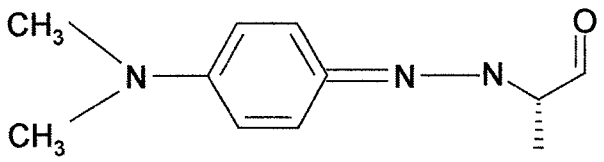


Figure 1.6. Structure of L-alanyl-DMPPD – a potential substrate for detection of L-alanyl aminopeptidase activity.

Potentially, 4-aminophenol (Fig 1.7) and its derivatives may couple with naphthol in a similar manner to DMPPD and form a coloured complex in a reaction equivalent to the "Nadi" reaction (Fig 1.8). By derivatisation of 4-aminophenol with an appropriate compound, this would produce potential substrates for detection of other taxonomically important enzymes.

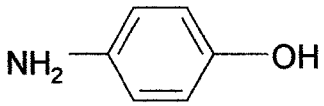


Figure 1.7 Structure of 4-aminophenol.

A significant advantage of 4-aminophenol over *p*-phenylenediamines is its ability to be derivatised with an amino acid at a position *para* to the hydroxyl group, and with a phosphate or carbohydrate moiety at the hydroxyl position, to produce substrates for aminopeptidases, phosphatases and glycosidases respectively. The substrate 4-aminophenol phosphate is commercially available, and has been used in enzyme flow immunoassays (Burestedt *et al.*, 2000) and as a substrate for the detection of progesterone in milk using an ampometric method (Pemberton *et al.*, 1999).

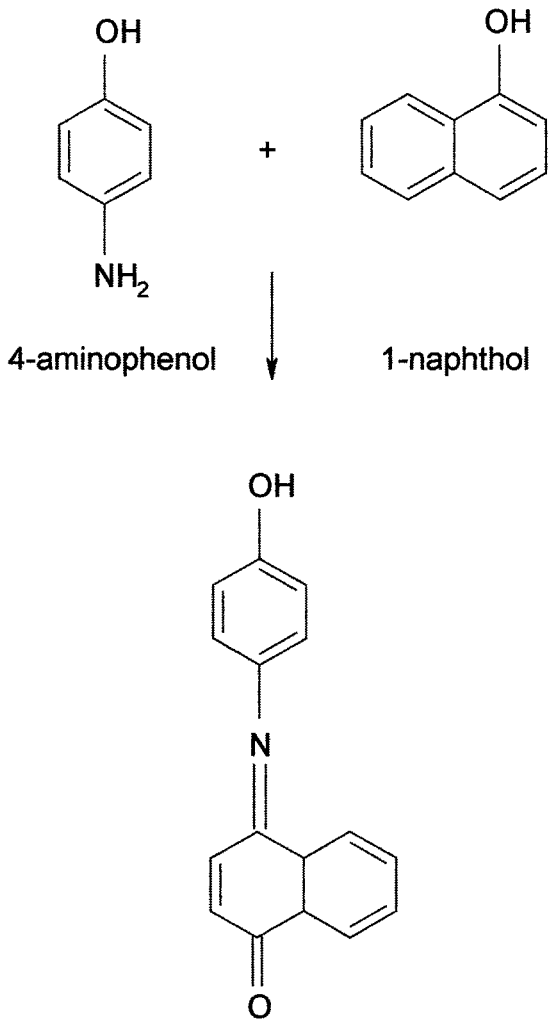


Figure 1.8. Structure of the coloured complex formed by the coupling of 4-aminophenol and 1-naphthol.

Rationale of the study

The literature reviewed in the previous section indicates that most of the available substrates for the detection of a range of bacterial hydrolyases suffer from one or more disadvantages. These include: diffusion through solid media; toxicity; high cost of synthesis: and the requirement for sensitive instrumentation for quantitative analysis.

The potential benefits of using derivatised 4-aminophenol and *p*-phenylenediamines as substrates for bacterial hydrolyases are substantial, with the opportunity to overcome the fundamental problems associated with traditional chromogenic and fluorogenic labels listed above. In the presence of an appropriate coupling agent, a wide variety of intensely coloured reaction products of 4-aminophenol and *p*-phenylenediamines might be obtained, depending on the coupling agent used. Furthermore, substrates produced for detection of bacterial glycosidases by derivitisation of the phenolic hydroxyl group of the coupling agent, e.g. 1-naphthol-β-D-galactoside, may allow the production of "dual substrate" systems for the simultaneous detection of two different enzyme systems. For example, when used in conjunction with L-alanyl-DMPPD or L-alanyl-4-aminophenol or other aminoacyl derivatives, generation of colour would only occur when both the peptidase and glucosidase enzymes are present in the target organisms, since the un-derivitised substrates do not react to generate

colour. The potential applications of such systems (Fig 1.9) are considerable.

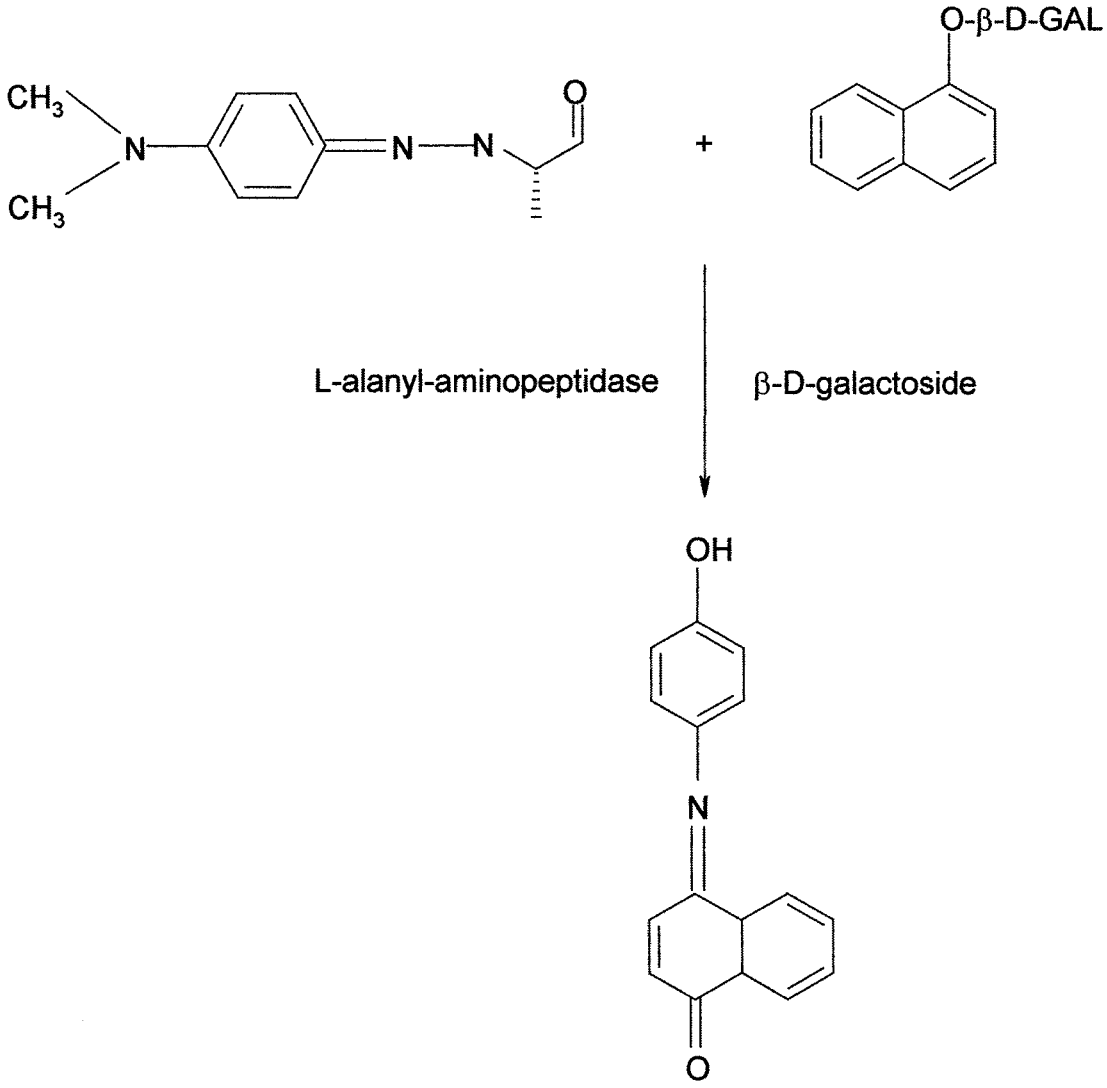


Figure 1.9. Reaction of L-alanyl-4-aminophenol and 1-naphthyl-β-D-galactoside showing the potential reaction product when both enzymes are present in a target organism.

It is also possible that other chemical compounds, with a structural similarity to 1-naphthol, may react with *p*-phenylenediamines to produce a range of

coloured reaction products. For example, compounds based on hydroxyanthraquinones (Abo *et al.*, 2001), hydroxyflavones (Fig 1.10), and pyrazolones might be useful.

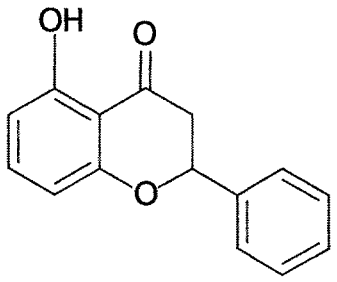


Figure 1.10. Chemical structure of hydroxyflavone. The reaction site with *p*-phenylenediamines or 4-aminophenol would be para to the hydroxyl group.

In addition, compounds which have relevant chemical groups e.g. OH, NH_2 etc., may form non-diffusible coloured chelates with metal ions, thus providing a range of substrates to rival indoxyl (Ley *et al.*, 1988), cyclohexeneoesculetin (James, 1998), and, more recently, alizarin (James, 2000a), and *p*-naphtholbenzein derivatives, (James, 2000b). Currently these expensive derivatives are the only commercially available non-diffusible substrates for bacterial identification in solid media.

Chapter 2

Investigation of the coupling of derivatives of 4-aminophenol and *p*-phenylenediamines with derivatives of 1-naphthol.

Introduction:

The initial focus of the project lay in demonstrating the coupling between *p*-phenylenediamines and 1-naphthol, i.e. validating the "Nadi reaction" (Keilin and Hartree, 1937; Burnstone, 1958). Moreover, it was necessary to establish that the coloured product formed on coupling can be suitably intense and therefore could be of use in the production of a relevant substrate (Dealler, 1993). Once this was established, it was possible to consider other compounds, which possess an underivatised position para to a suitably reactive group on a benzene ring as potentially useful alternatives for further study e.g hydroxyflavones (Fig 1.10), dihydroxyphenols (Fig 2.1), and hydroxyanthraquinones (James *et al.*, 2000a).

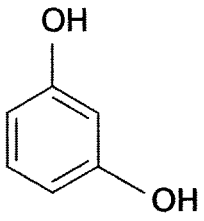


Figure 2.1 Chemical structure of 1,3-dihydroxyphenol (resorcinol), a potential compound for coupling with *p*-phenylenediamines and aminophenols.

For example, 4-aminophenol and its derivatives, which on coupling with phenols and/or naphthols also produced highly-coloured compounds,

especially when the halogenated derivatives of 4-aminophenol are used (Clark, 1972). The products formed by these compounds in coupling with phenols and naphthols have previously been investigated as possible pH indicators – they include simple indophenols, more complex naphthone indophenols and their derivatives (Clark, 1972). Most, however, were found to be unsuitable due to low solubility or instability. Therefore of prime importance in the early stages of the present study was the production of a sensitive and specific reaction system with easily determinable and stable reaction products, by considering a range of derivatives of naphthols, aminophenols, and *p*-phenylenediamines or other compounds to satisfy these requirements.

The complexes formed upon coupling of *p*-phenylenediamines, 4-aminophenols and derivatives of 1-naphthol are highly coloured indamine and indophenol dyes (Burnstone, 1958). Coupling occurs instantaneously since all of the chemical species involved are of high energy, and are thus highly reactive. Upon coupling a relatively low energy, non-reactive, conjugated system is formed (Finar, 1973). Compounds with extended conjugated systems are frequently highly coloured and often used as dyes or indicators (Lister and Renshaw, 1991). The dye colour is produced by absorption of energy by the bonding electrons on atoms such as nitrogen and oxygen, although other atoms are also involved. Indophenol blue (Fig. 1.4), formed by the coupling of DEPPD and 1-naphthol has a highly

delocalised system of alternating single and double bonds associated mainly with its aromatic structure. This is a conjugated system, which is often associated with colour (Finar, 1973). The compound absorbs light of a particular wavelength when electrons are promoted to higher energy levels. Non-absorbed light is transmitted, resulting in the observed colour. A good example of this type of conjugated structure is the pH indicator methyl orange, which is yellow at alkaline pH, and red at acid pH, due to acceptance of a proton (Lister and Renshaw, 1991). This affects the compound's conjugated system and alters both light absorption, and transmission. However, dyes such as indophenol blue cannot accept protons in this way and therefore do not change colour upon pH variation.

In contrast to indophenol blue, the complex formed by coupling of 4-aminophenols with 1-naphthol, differs in that the diethyl-amino group is replaced by a hydroxyl group (Fig 1.8). This is likely to have an effect on the delocalisation of electrons within the conjugated system and, therefore on both the absorption and transmission of light. It was envisaged that derivatives of *p*-phenylenediamines and 4-aminophenols might produce a variety of different coloured compounds when coupled with 1-naphthol or derivatives, due to the different conjugated systems produced upon coupling. The most important of these were further investigated as candidates for production of chromogenic substrates, as described in this Chapter.

Materials and Methods

Cultures

All bacteria used in this Chapter were National Collection of Type Culture control strains purchased from Central Public Health Laboratory, Colindale, London. All organisms were freshly subcultured for use in all experiments on Columbia agar base supplemented with 5% v/v horse blood and checked for purity before experimental use. The Gram-negative control strains were *Escherichia coli* NCTC 10418, *Klebsiella pneumoniae* NCTC 10896, *Enterobacter cloacae* NCTC 11936, *Serratia marcescens* NCTC 10211, *Salmonella typhimurium* NCTC 74. The Gram-positive control strains used were *Enterococcus faecalis* NCTC 755, and *Staphylococcus aureus* NCTC 6571.

Chemicals

DMPPD (monohydrochloride), DEPPD (monohydrochloride), N,N,N,N-tetramethyl-*p*-phenylenediamine (TMPPD), 4-amino-2,6-dichlorophenol, 4-amino-2,6-dibromophenol, sodium hydroxide, 1-naphthol, 4-aminophenol (hydrochloride), 3,5-dihydroxy-2-naphthoic acid, 4-chloro-1-naphthol, 5-{[(1-hydroxy-2-naphthyl)carbonyl]octadecylamino}isophthalic acid, anthranol, phloroglucinol anhydrous, naphthol AS-IRG, 3-phenyl-5-isoxazolone, 3-methyl-2-benzothiazolone hydrazone hydrochloride, resorcinol, 3-methyl-1-phenyl-2-pyrazoline-5-one, barbituric acid, rhodanine-3-acetic acid, and di-isoproterenol dihydrochloride were obtained from the Sigma Aldrich Chemical Company Ltd, Poole, UK.

Media

Columbia agar base (Oxoid Ltd, Basingstoke, Hampshire.) was prepared by dissolving 39 g of powder in 1 litre of de-ionised water. The medium was sterilised by autoclave at 114°C for 20 minutes and cooled to 50°C in a waterbath. When cooled 5% v/v sterile horse blood was added (TCS Biosciences Ltd, Botolph, Claydon, Buckinghamshire). Sterile Brain Heart Infusion broth (Oxoid) was prepared by dissolving 37 g of powder in 1 litre of de-ionised water and sterilised by autoclave at 114°C for 20 minutes. Phosphate buffer A was prepared by dissolving 15.6 g of sodium dihydrogen phosphate (BDH:Analar) in 500 ml of de-ionised water. Phosphate buffer B was prepared by dissolving 14.195 g of di-sodium hydrogen phosphate (BDH:Analar) in 500 ml of de-ionised water. Both solutions were sterilised by autoclave at 114°C for 20 minutes. Phosphate buffer used in all experiments was prepared by adding 28 ml of solution A and 72 ml of solution B into a sterile Duran bottle. One hundred ml of sterile de-ionised water was then added. This produced a 0.1 mol l^{-1} phosphate buffer solution at pH 7.2.

Substrates

All chromogenic substrates were synthesised by Dr A.L. James, University of Northumbria.

Equipment

All compounds were weighed out using a Sartorius 2434 electronic balance; - accurate to 0.1 mg. (Sartorius Limited, Epsom, UK). Semi-automatic Gilson pipettes (P200 and P1000) with sterile disposable tips were used in all experiments (Gilson Medical Electronics, Villiers-le-Bel, France). Sterile flat bottom microtitre plates (Bibby Sterilin Limited, Aberbargoed, UK) were used and incubated in a LEEC 37°C shaking incubator (Laboratory and Electrical Engineering Company, Nottingham, UK).

Absorbance spectra were prepared using an ATIUnicam-UV/Vis scanning double beam spectrophotometer (Unicam Ltd, York Street, Cambridge, UK).

Demonstration of the coloured complex formed between *p*-phenylenediamines and 1-naphthol.

Individual 5 mmol l^{-1} stock solutions of the following compounds were prepared by dissolving either 8.6 mg of DMPPD (monohydrochloride), 9 mg of DEPPD (monohydrochloride), 11.9 mg of TMPPD, or 7.2 mg of 1-naphthol in 1 ml of 0.1mol l^{-1} NaOH. After dissolving the solutions were made up to 10 ml volume by adding 9 ml of phosphate buffer, and the pH was then adjusted to 7.2.

A 50 µl sample of each *p*-phenylenediamine solution was then added to 50 µl of 1-naphthol solution in a microtitre plate. A 50 µl volume of each stock solution and 50 µl of phosphate buffer were used as controls. The plate was placed into a shaking incubator and examined visually after four hours for the production of a coloured reaction product.

Demonstration of the coloured complex formed between 4-aminophenol, 4-amino-2,6-dibromophenol, or 4-amino-2,6-dichlorophenol and 1-naphthol.

Stock solutions of the following were prepared at 5 mmol l^{-1} by dissolving either 7.3 mg of 4-aminophenol; 8.9 mg of 4-amino-2,6-dichlorophenol; 13.3 mg of 4-amino-2,6-dibromophenol; or 7.2 mg of 1-naphthol in 1 ml of 0.1 mol l^{-1} NaOH. After dissolving the solutions were made up to 10 ml volume by adding 9 ml of phosphate buffer, and the pH was then adjusted to 7.2.

A 50 µl aliquot of each aminophenol solution was then added to 50 µl of 1-naphthol solution in a microtitre plate. A 50 µl volume of each stock solution and 50 µl of phosphate buffer were used as controls. The plate was placed into a shaking incubator and examined visually after four hours for the production of a coloured reaction product

Investigation of the colour produced by reaction of *p*-phenylenediamines, and 4-aminophenols with derivatives of 1-naphthol.

Individual 5 mmol l^{-1} stock solutions of DEPPD, DMPPD, 4-aminophenol, 4-amino-2,6-dichlorophenol, and 4-amino-2,6-dibromophenol were prepared as described previously. Stock solutions of five naphthol derivatives were prepared by dissolving the following in individual 1 ml volumes of 0.1 mol l^{-1} NaOH: 7.2 mg of 1-naphthol; 10.2 mg of 3,5-dihydroxy-2-naphthoic acid; 8.9 mg of 4-chloro-1-naphthol; 9.2 mg of anthranol and 30.2 mg of 5-{[(1-hydroxy-2 naphthyl)carbonyl]octadecylamino}isophthalic acid. Upon dissolving the solutions were made up to 10 ml volume by adding 9 ml of phosphate buffer, and the pH was then adjusted to 7.2.

A 50 µl sample of each *p*-phenylenediamine or aminophenol solution was then added to 50 µl of each naphthol solution in a microtitre plate. A 50 µl volume of each solution and 50 µl of phosphate buffer were used as controls. The plate was placed into a shaking incubator and examined visually after four hours for the production of a coloured reaction product.

Investigation of the optimal pH for the production of a coloured complex using DEPPD and 4-aminophenol coupled with 1-naphthol and 3,5-dihydroxy-2-naphthoic acid.

Stock solutions of the following were prepared at 5 mmol l^{-1} by dissolving either 7.3 mg of 4-aminophenol, 9 mg of DEPPD, 7.2 mg of 1-naphthol, or 10.2 mg of 3,5-dihydroxy-2-naphthoic acid in 1 ml of 0.1 mol l^{-1} NaOH. These were added to 9 ml of phosphate buffer, prepared at pH 6.0–7.6 in 0.2 pH increments. Using a Gilson pipette 50 µl of each either DEPPD or 4-aminophenol was added to a separate well of a microtitre plate and mixed with 50 µl of each naphthol solution at the same pH. This was repeated for all compounds at each pH. The plate was incubated at 37°C in a shaking incubator and examined visibly after 4 hours for colour production. As a control 50 µl of each core compound was mixed with an equal volume of phosphate buffer.

Determination of toxicity of the core compounds, 4-aminophenol, 4-amino-2,6-dibromophenol, 4-amino-2,6-dichlorophenol, DMPPD, and DEPPD against Gram-positive and Gram-negative bacteria.

Each of the five core compounds was prepared at a stock concentration of 200 mmol l^{-1} by dissolving 34.5 mg of DMPPD, 36.2 mg of DEPPD, 29.1 mg of 4-aminophenol, 35.6 mg of 4-amino-2,6-dichlorophenol and 50.4 mg

of 4-amino-2,6-dibromophenol in 1 ml of 0.1 mol l^{-1} NaOH. Each of these stock solutions was then diluted 1/20 in sterile brain-heart infusion broth (BHI) to produce 10 mmol l^{-1} solutions. When dissolved, the pH was adjusted to 7.2 and the solution was filter sterilised. Serial double dilutions of each core molecule were then prepared ranging from 10 mmol l^{-1} to 0.156 mmol l^{-1}.

The seven NCTC control strains listed previously were subcultured onto Columbia blood agar plates. Growth of each organism was harvested after 18-hour incubation at 37°C and suspended in 10 ml of sterile BHI broth to McFarland standard of 1.0 using an API Densimat. The concentration of organisms was found to be approximately 10^6 organisms ml^{-1} by serial dilution of the suspension on Columbia blood agar plates. A 10 µl aliquot of each organism suspension was then added to 90 µl of each dilution of core molecule. A growth control without substrate was included for each organism. Growth was determined by measuring the absorbance of each well at 690 nm every 30 minutes for five hours.

Determination of the toxicity of various naphthol derivatives against both Gram-positive and Gram-negative bacteria.

The following naphthols were weighed out to produce stock solutions of 200 mmol l^{-1} by dissolving 40.8 mg of 3,5-dihydroxy-2-naphthoic acid,

47.8 mg of 3,5-dihydroxynaphthalene-2-carbonyl-2,4,6-trimethylanilide, 36.6 mg of anthranol, 120.8 mg of 5[[(1-hydroxy-2-naphthyl)-carbonyl]octadecylamino]-isophthalic acid, 28.8 mg of 1-naphthol and 35.7 mg of 4-chloro-1-naphthol in 1ml of 0.1 mmol l^{-1} sodium hydroxide: When dissolved 100 µl was added to 9.9 ml of BHI broth (pH 7.2). The pH was re-adjusted to 7.2 and filter-sterilised. This produced a test solution of 2 mmol l^{-1}. This solution was then double-diluted through BHI broth to produce a concentration series from 2 mmol l^{-1} - 0.125 mmol l^{-1}.

The seven NCTC control strains listed previously were subcultured onto Columbia blood agar plates grown and harvested as described above. A 10 µl aliquot of each organism suspension (approximately 10^6 organisms ml^{-1}) was then added to 90 µl of each dilution of naphthol. A growth control without substrate was included for each organism. Growth was determined by measuring the absorbance of each well at 690 nm every 30 minutes for five hours.

Determination of the toxicity of various L-alanyl substrates against both Gram-positive and Gram-negative bacteria.

Following the toxicity assessment of naphthols, aminophenols and *p*-phenylenediamine compounds, it was decided to examine the toxicity of L-alanyl-based substrates as representative of the chromogenic substrates. Therefore, L-alanyl derivatives of DMPPD, DEPPD, 4-aminophenol, 4-amino-2,6-dibromophenol and 4-amino-2,6-dichlorophenol were tested against both Gram-positive and Gram-negative organisms. All 5 substrates were dissolved in 1 ml of sterile de-ionised water and diluted further in 9 ml of BHI broth. The solution was sterilised by membrane filtration. This produced a concentration range from 1.25 mmol l^{-1} to 0.078 mmol l^{-1}.

The seven NCTC control strains were sub-cultured, grown and suspended in sterile BHI broth at 10^6 organisms ml^{-1} as listed previously were subcultured onto Columbia blood agar plates. Growth of each organism was harvested after 18-hours at 37°C and suspended in 10 ml of sterile BHI broth to McFarland standard 1.0 using an API densimat. A10 µl aliquot of each organism suspension was then added to 90 µl of each substrate dilution. A growth control without substrate was included for each organism. Growth was determined by measuring the absorbance of each well at 690 nm every 30 minutes for five hours.

Determination of the optimal conditions for colour development produced by *E.coli* in the presence of L-alanyl-DEPPD and 3,5-dihydroxy-2 naphthoic acid.

A number of different variables were investigated to determine the conditions for optimal colour development generated by *E.coli* in the presence of these two constituents. The experiment was essentially a "chequerboard titration" in which a range of concentrations of 3,5-dihydroxy-2-naphthoic acid solutions were tested against a range of concentrations of L-alanyl-DEPPD and incubated with standard innocula of *E.coli*. The final concentration range of the naphthol was 0.078-5 mmol l^{-1}. The final concentration range of L-alanyl-DEPPD was 0.0156 mmol l^{-1}-2.5 mmol l^{-1}. Twelve different chequerboard titrations were prepared in total using three different inocula of *E.coli* (final concentrations: 4 x 10^8, 8 x 10^8, 10^9 cfu ml^{-1} and uninoculated control well). Also, the volume of the reaction mixture was varied (50 µl, 100 µl and 200 µl) to investigate the effect of volume on the intensity of the colour formation. All reactions were performed in phosphate buffer.

All test reactions were incubated at 37°C for four hours. At half hourly intervals all wells were read for absorbance at 620 nm and 405 nm. When the data were analysed, 405 nm readings were subtracted from 620 nm

readings to give an accurate measurement of indamine formation (620 nm) corrected for increases in turbidity (405 nm) due to organism growth.

Determination of the optimal conditions for colour development produced by a range of organisms in the presence of L-alanyl-DEPPD and 3,5-dihydroxy-2-naphthoic acid.

The above experiment was repeated using the seven NCTC control strains to determine the optimal concentrations of the reactants; i.e. L-alanyl-DEPPD and 3,5-dihydroxy-2-naphthoic acid. In view of the findings of the previous experiment, all strains were tested at a single inoculum (final concentration: approximately 4 x 10^8 cfu ml^{-1}) with an overall reaction volume of 200 µl.

Determination of the colour produced by *E.coli* in the presence of five L-alanyl and six naphthol derivatives.

The following naphthols were prepared at 2.5 mmol l^{-1} by dissolving 5.1 mg of 3,5-dihydroxy-2-naphthoic acid, 15.1 mg of 5[[(1-hydroxy-2-naphthyl) carbonyl]octadecylamino]-isophthalic acid, 6 mg of 3,5-dihydroxynaphthalene-2-carbonyl-2,4,6-trimethylanilide, 3.6 mg of 1-naphthol, 4.6 mg of anthranol, and 4.5 mg of 4-chloro-1-naphthol in 1ml

of 0.1 mol l^{-1} NaOH and then diluting in 9 ml of phosphate buffer; The final pH was adjusted to 7.2 and each solution was filter sterilised. L-alanyl derivatives of DMPPD, DEPPD, 4-aminophenol, 4-amino-2,6-dichlorophenol and 4-amino-2,6-dibromophenol were prepared at 10 mmol l^{-1} in phosphate buffer. The final pH was adjusted to 7.2 and each was filter sterilised.

A volume of 50 µl of each alanyl derivative was placed in six wells of a microtitre plate and 50 µl of one or other of each naphthol derivative was then added. 100 µl of an *E.coli* suspension (NCTC 10418: 4 x 10^8 cfu ml^{-1}) was added and the plate was then incubated in a shaking incubator at 37°C. The final concentrations of naphthols and L-alanyl derivatives were 0.625 mmol l^{-1} and 2.5 mmol l^{-1} respectively. Results were recorded visibly after four hours incubation.

Determination of the optimal concentration of 3,5-dihydroxy-2-naphthoic acid for the production of colour using 4-aminophenyl-β-D-glucuronide.

A "chequerboard" titration was carried out with a range of concentrations of 3,5-dihydroxy-2-naphthoic acid tested against a range of concentrations of 4-aminophenyl-β-D-glucuronide in the presence of *E.coli* (NCTC 10418).

An 80 mmol l^{-1} stock solution of 3,5-dihydroxy-2-naphthoic acid was prepared by dissolving 16.3 mg in 1 ml of 0.1 mol l^{-1} NaOH and diluting in 9 ml of phosphate buffer. The pH was adjusted to 7.2 and sterilised by membrane filtration. A 20 mmol l^{-1} stock solution of 4-aminophenol-β-D-glucuronide was prepared by dissolving 57.9 mg in 10 ml of phosphate buffer. The pH was adjusted to 7.2 and the solution sterilised by membrane filtration. The β-D-glucuronide solution was double diluted in phosphate buffer to produce a concentration range of 10-1.25 mmol l^{-1}.

Into each well of a microtitre plate, a 50 µl volume of stock 3,5-dihydroxy-2-naphthoic acid solution was double diluted in phosphate buffer, and 50 µl of each 4-aminophenyl-β-D-glucuronide dilution added. An aliquot of 100 µl of an *E.coli* suspension (NCTC 10418: 4 x 10^8 cfu ml^{-1}) was added to all wells. This produced a final concentration range of 20-0.3125 mmol l^{-1} for 3,5-dihydroxy-2-naphthoic acid and 5-0.3125 mmol l^{-1} for 4-aminophenyl-β-D-glucuronide and an initial inoculum of 2x10^8 cfu ml^{-1}. Results were recorded visibly after overnight incubation at 37°C.

Production of absorption spectra for the various complexes formed between aminophenol, *p*-phenylenediamines and naphthols.

Spectra were produced for the indamine/indophenol complexes formed between 4-aminophenol, 4-amino-2,6-dichlorophenol, DEPPD, and DMPPD in reaction with 3,5-dihydroxy-2-naphthoic acid and 1-naphthol.

A 5mmol l^{-1} stock solutions of DEPPD, DMPPD, 4-aminophenol, 4-amino-2,6-dichlorophenol, and 4-amino-2,6-dibromophenol was prepared as described previously. Stock solutions (5 mmol l^{-1}) of naphthol derivatives were prepared by dissolving 7.2 mg of 1-naphthol, 10.2 mg of 3,5-dihydroxy-2-naphthoic acid, 8.9 mg of 4-chloro-1-naphthol and 30.2 mg of 5-{[(1-hydroxy-2-naphthyl)carbonyl]octadecylamino}isophthalic acid in 1 ml of 0.1 mmol l^{-1} NaOH. Upon dissolution the solutions were made up to volume by adding 9 ml of phosphate buffer, and the pH adjusted to 7.4.

A volume of 1 ml of each phenol derivative stock solution was mixed with 1 ml of each naphthol stock solution in a sterile plastic universal container. The solution was incubated at 37°C for 4 hours in a shaking water bath to allow the components to react. Each coloured solution was diluted 1:100 in phosphate buffer (pH 7.2) and absorption spectra produced. The blank for each spectrophotometric measurement was phosphate buffer.

Evaluation of a range of compounds as alternatives to naphthol for the formation of coloured complexes with DEPPD.

A 5 mmol l^{-1} solution of the following were prepared by dissolving in 1 ml of 0.1 mol l^{-1} NaOH: - 6.3 mg of phloroglucinol (anhydrous), 13.6 mg of naphthol AS-IRG, 8.0 mg of 3-phenyl-5-isoxazolone, 10.8 mg of 3-methyl-2-benzothiazolone hydrazone hydrochloride, 5.5 mg of resorcinol, 8.7 mg of 3-methyl-1-phenyl-2-pyrazoline-5-one, 6.4 mg of barbituric acid, 6.7 mg of rhodanine-3-acetic acid, or 17.3 mg of di-isoproterenol dihydrochloride, in 1 ml of 0.1 mol l^{-1} NaOH followed by 9 ml of phosphate buffer. The pH was re-adjusted to 7.2 and filter-sterilised.

A 5 mmol l^{-1} solution of DEPPD was prepared by dissolving 9.0 mg in 1 ml of 0.1 mol l^{-1} NaOH and making up to volume using phosphate buffer. The pH was adjusted to 7.2 and the solution filter-sterilised.

A volume of 50 µl of each test solution was placed into an individual well of a microtitre plate and 50 µl of DEPPD added. The plate was placed into a shaking incubator and examined after 4 hours incubation for the presence of a coloured reaction product. As controls, each individual compound and DEPPD was incubated with an equal volume of phosphate buffer.

RESULTS

Demonstration of the coloured complex formed between *p*-phenylenediamines and 1-naphthol.

All three *p*-phenylenediamine derivatives produced a deep blue colour in the wells containing 1-naphthol (Fig 2.2). The control wells for DEPPD and DMPPD were a pink/red colour. The tetramethyl derivative produced an intense blue colour in the presence of naphthol. However a similar colour was observed in the presence of buffer alone. This would suggest that this derivative is unsuitable for demonstrating coupling between *p*-phenylenediamines and 1-naphthol since an indamine dye is formed irrespective of the coupling conditions. In particular, if derivatised naptholics were used for coupling with free TMPPD a blue coloured product would develop without the requirement for substrate hydrolysis. Additionally, TMPPD has no deriviatisable groups suitable for the production of substrates. Thus DEPPD and DMPPD would appear to offer the greatest potential for derivatisation as chromogenic substrates in a variety of formats.

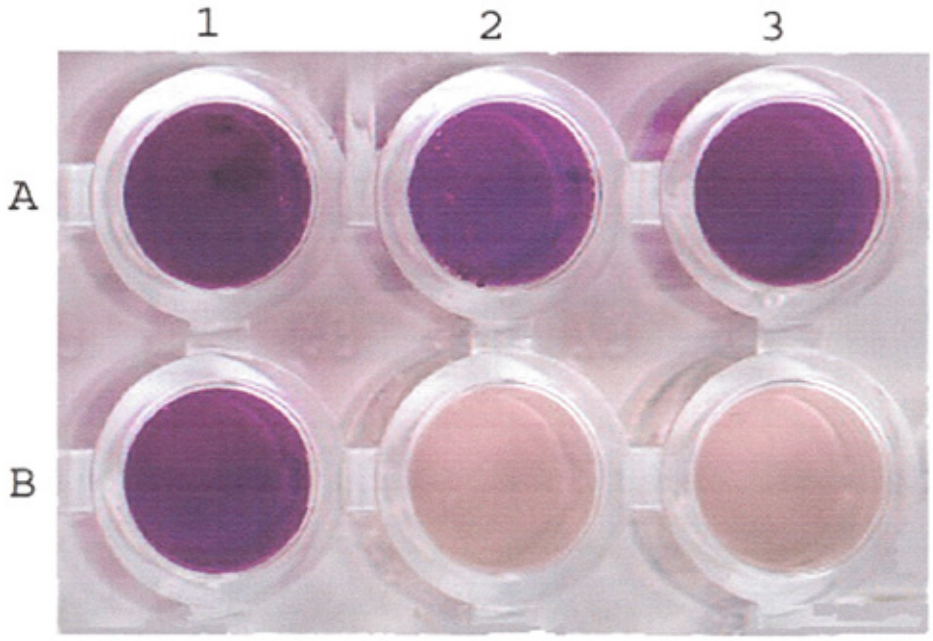


Figure 2.2 Reaction of TMPPD (A:1), DEPPD (A:2) and DMPPD (A:3) and 1-naphthol. Control wells B:1- B:3 contain each *p*-phenylenediamine and buffer only.

Demonstration of the coloured complex formed between 4-aminophenol, 4-amino-2,6-dibromophenol, or 4-amino-2,6-dichlorophenol and 1-naphthol.

The halogenated 4-aminophenol derivatives produced a deep red colour (Fig 2.3) with the formation of a precipitate, upon coupling with 1-naphthol (wells C:7 and C:9). The un-substituted 4-aminophenol produced an orange coloured complex (well C:5). Little or no colour was observed in control wells. However, despite warming, the di-bromo compound was less soluble in phosphate buffer than the chlorinated derivative. Upon standing the compound was also observed to form a slight precipitate. Overall the colour produced by both halogenated derivatives was equally intense, except in wells containing anthranol (wells D:7 and D:9), with the di-bromo compound producing only a very weak orange colour. Thus the increased solubility of the di-chloro and the stronger reaction with anthranol (D:7), suggests a clear advantage over the bromonated compound. These results would suggest that both 4-aminophenol and its halogenated derivatives would be useful adjuncts to *p*-phenylenediamines for the production of coloured complexes with 1-naphthol since they may offer opportunities for subsequent derivatisation as range of substrates.

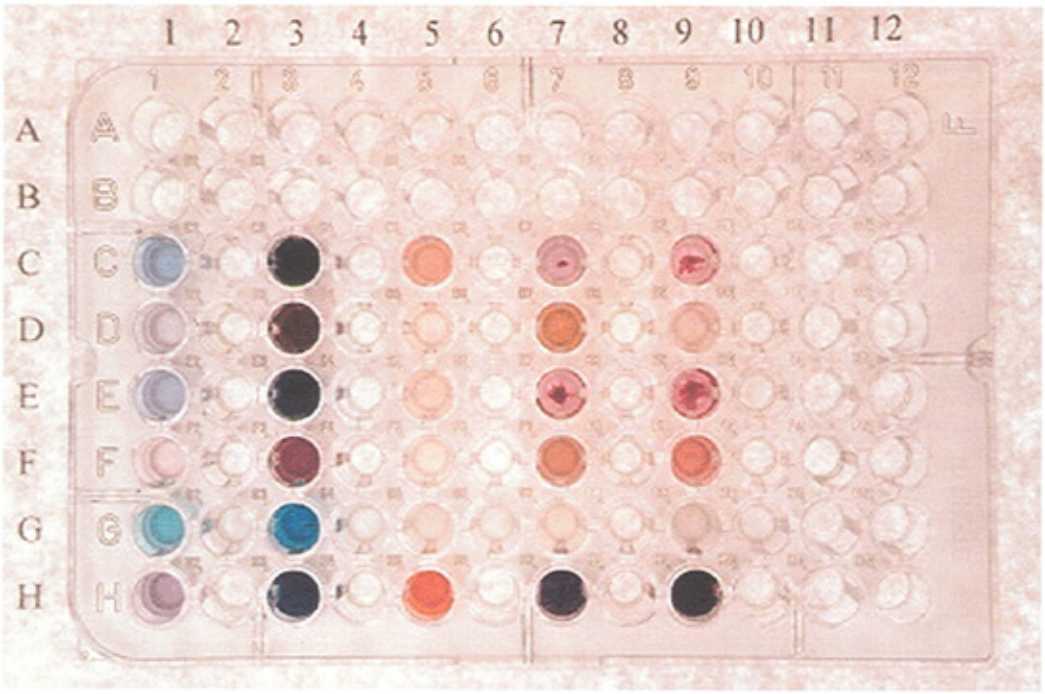


Figure 2.3. The colour development of six naphthols (0.625 mmol l^{-1}) in the presence of five core compounds (2.5 mmol l^{-1}).

Row C	1-naphthol
Row D	anthranol
Row E	4-chloro-1-naphthol
Row F	3,5-dihydroxynapthalene-2-carbonyl-2,4,6-trimethylanilide
Row G	5[[(1-hydroxy-2-naphthyl)-carbonyl]octadecylamino]-isophthalic acid
Row H	3,5-dihydroxy-2-naphthoic acid

Column 1	DMPPD
Column 3	DEPPD
Column 5	4-aminophenol
Column 7	4-amino-2,6-dichlorophenol
Column 9	4-amino,2,6-dibromophenol

(Even numbered columns contain buffer only)

Investigation of the colour produced by reaction of *p*-phenylenediamines, and 4-aminophenols with derivatives of 1-naphthol.

The results of this experiment are also shown in Figure 2.3. Of the two *p*-phenylenediamine derivatives the strongest reaction occurred visibly with the diethyl derivative for all of the naphthols tested (column 3). The most intense colours developed with DEPPD in coupling with 1-naphthol (C:3), 4-chloro-1-naphthol (E:3), 5[[(1-hydroxy-2-naphthyl)-carbonyl]octadecylamino]-isophthalic acid (G:3) and 3,5-dihydroxy-2-naphthoic acid (H:3), Overall, DMPPD produced weaker reactions (column 1) than the diethyl derivative (column 3), although this may have been attributable to the poorer solubility of the former compound. Despite warming the compound always partially came out of solution. It was interesting to note that 5-[[(1-hydroxy-2-naphthyl)-carbonyl]octadecylamino]-isophthalic acid (row G) only reacted with the two *p*-phenylenediamines producing a turquoise complex (wells G:1 and G:3). For the 4-aminophenol derivatives tested, 4-aminophenol itself (colunm 5) produced a strong reaction in the presence of both 3,5-dihydroxy-2-naphthoic acid (H:5), and 1-naphthol (C:5), and a less intense reaction product with 4-chloro-1-naphthol (E:5). For the dibromo and dichloro derivatives, strong reactions were recorded with 3,5-dihydroxy-2-naphthoic acid (H: 7 and H: 9), 1-naphthol (C: 7 and C: 9)

and 4-chloro-1-naphthol (E: 7 and E: 9), reaction with the latter two naphthols forming red/purple precipitates in the well. No colour was observed in the wells containing naphthol plus buffer alone.

Based on these results it is clear that the best naphthol derivatives for use with any 4-aminophenol were either 1-naphthol or 3,5-dihydroxy-2-naphthoic acid. The *p*-phenylenediamine substrates reacted well with several naphthols. Since the most intense of these reactions occurred with 3,5-dihydroxy-2-naphthoic acid and 1-naphthol, both can be considered highly useful for the development of coloured products using any of the 4-aminophenols and *p*-phenylenediamines examined.

Investigation of the optimal pH for the production of a coloured complex using DEPPD and 4-aminophenol coupled with 1-naphthol and 3,5-dihydroxy-2-naphthoic acid.

For both core compounds examined, colour was weakest at both extremes of pH tested i.e. 6.6 and 7.6 (Fig 2.4).

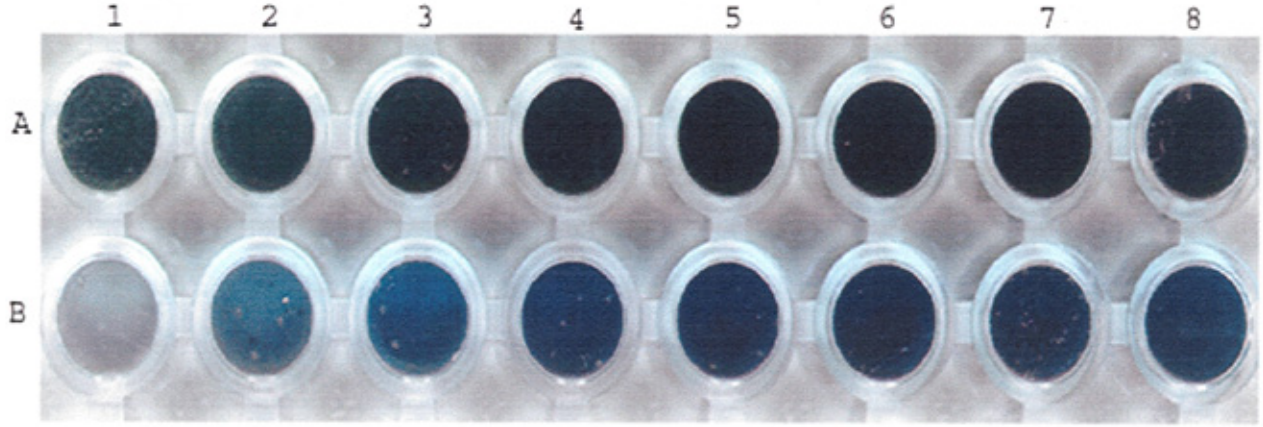


Figure 2.4. Colour development using DEPPD and either 1-naphthol or 3,5-dihydroxy-2-naphthoic acid at different pH, from 6.4 to 7.8 in 0.2 pH units. Top row shows DEPPD plus 3,5-dihydroxy-2-naphthoic acid (A:1-A:8). Bottom row shows DEPPD plus 1-naphthol (B:1-B:8). From pH 6.8 to 7.4, there was little difference in the intensity of the coloured reaction product. Since most culture media are prepared, at pH 7.2, all further experimental work with these compounds was carried out at this pH.

Determination of toxicity of the core compounds, 4-aminophenol, 4-amino-2,6-dibromophenol, 4-amino-2,6-dichlorophenol, DMPPD, and DEPPD against Gram-positive and Gram-negative bacteria.

The complete results for this experiment are shown in Appendix 2.1. It should be noted that at concentrations above 0.313 mmol l^{-1}, all core compounds generated a coloured product irrespective of the presence of organisms, thus interfering with spectrophotometric measurements of growth, therefore, graphs are shown only for concentrations from 0.078 mmol l^{-1} to 0.313 mmol l^{-1}. No increase in absorbance at 690 nm was observed in the inoculum free wells (BHI control). The BHI control showed an initial lag phase of 180 minutes, followed by a rapid increase in absorbance at 690 nm. The growth of *E.coli* (NCTC 10418) in the presence of 4-aminophenol is shown above in Figure 2.5.1, whilst the growth of *S.aureus* is shown in Figure 2.5.7.

The growth of *E.coli,* as judged by an increase in absorbance at 690 nm, was severely inhibited by 4-aminophenol at concentrations from 0.078 - 0.313 mmol l^{-1} (Fig 2.5.1). For the halogenated derivatives the di-bromo derivative (Fig 2.5.2) was less toxic than the dichloro derivative (Fig 2.5.3). DEPPD (Fig 2.5.4) inhibited the growth of *E.coli* more than DMPPD (Fig 2.5.5). Although both *p*-phenylenediamines were toxic, this was less than that observed with 4-aminophenol. For the other Gram-negative strains

tested, 4-aminophenol was highly toxic to all strains e.g. Figure 2.5.6 shows data for *S.marcescens*. In addition the toxicity of the halogenated derivatives was similar to that observed with *E.coli* i.e. the di-bromo derivative was less toxic than the di-chloro compound (Appendix 2.1). Of the Gram-positive strains examined, growth of *S.aureus* was severely inhibited by all of the five core compounds tested, with virtually no growth observed over 300 minutes in the wells containing 4-aminophenol (Fig 2.5.7). *E. faecalis* (NCTC 755) was inhibited, like all of the other strains tested, by 4-aminophenol (Fig 2.5.8) and 4-amino-2,6-dichlorophenol, with the di-bromo derivative being the least toxic of all the aminophenols examined (Appendix 2.1). For the *p*-phenylenediamines tested DMPPD inhibited the growth of *E.faecalis* less than DEPPD (Appendix 2.1).

Figure 2.5.1: Growth of *E.coli* in the presence of various concentrations of 4-aminophenol.

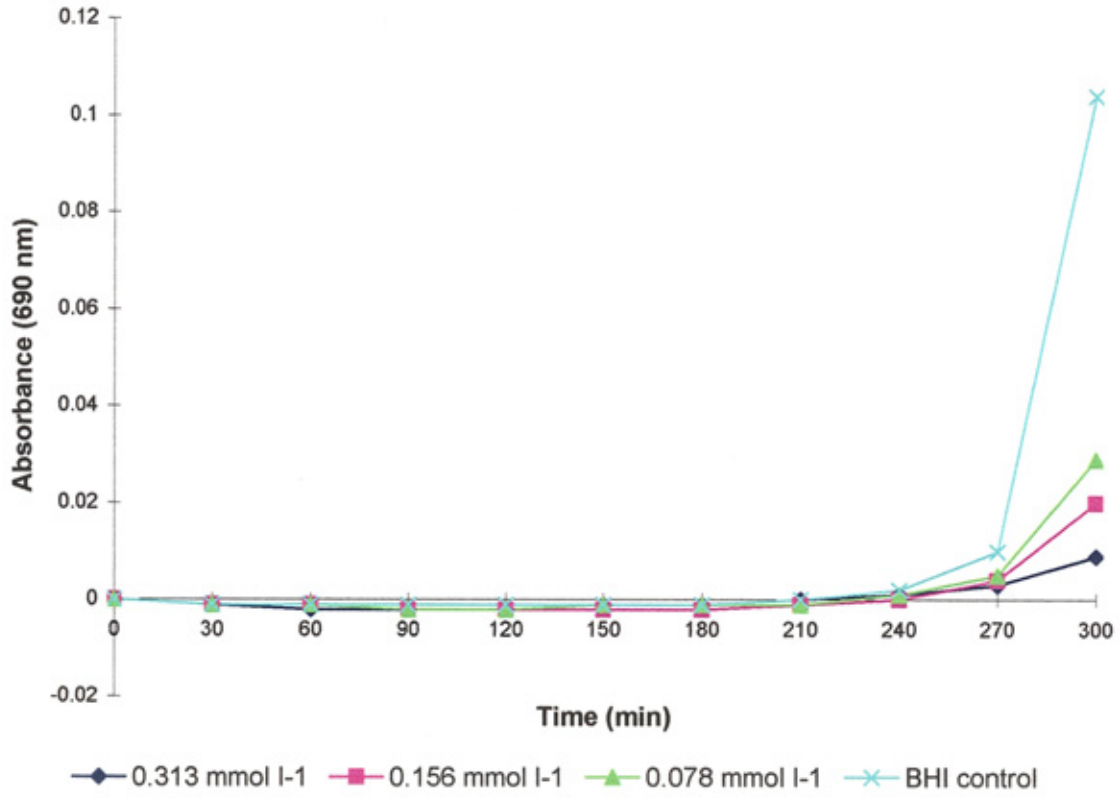


Figure 2.5.2: Growth of E.coli in the presence of various concentrations of 4-amino-2,6-dibromophenol.

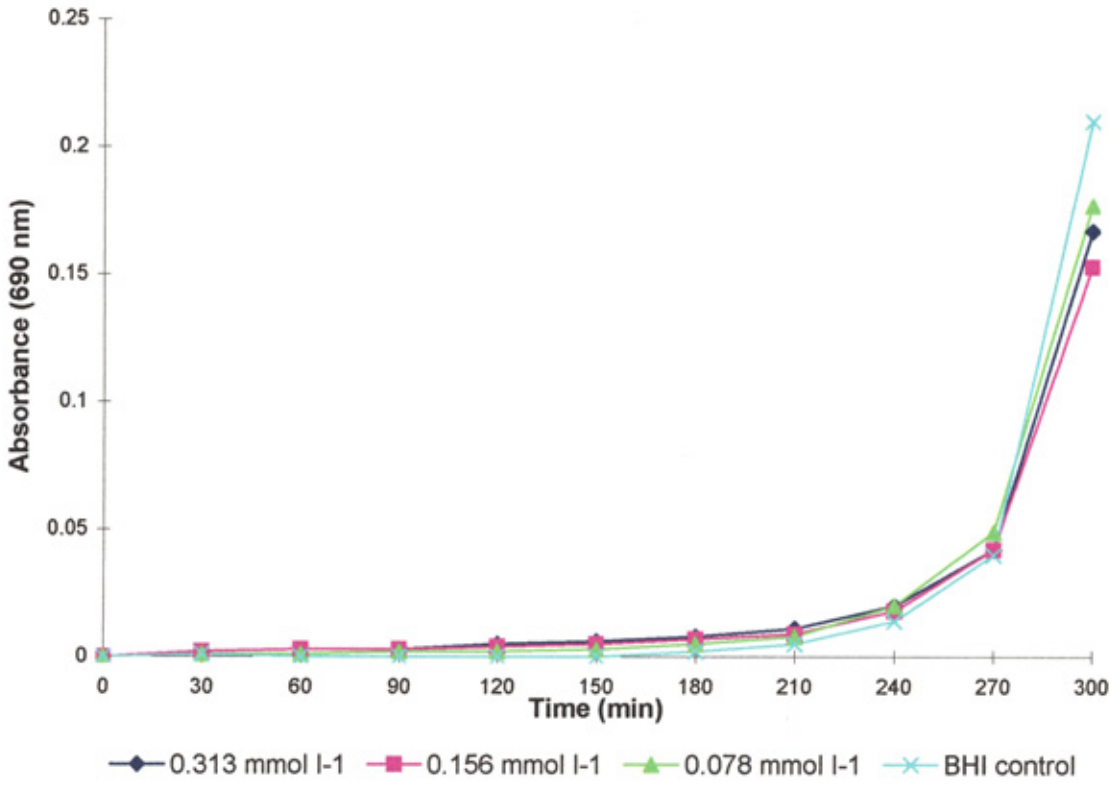


Figure 2.5.3: Growth of E.coli in the presence of various concentrations of 4-amino-2,6-dichlorophenol.

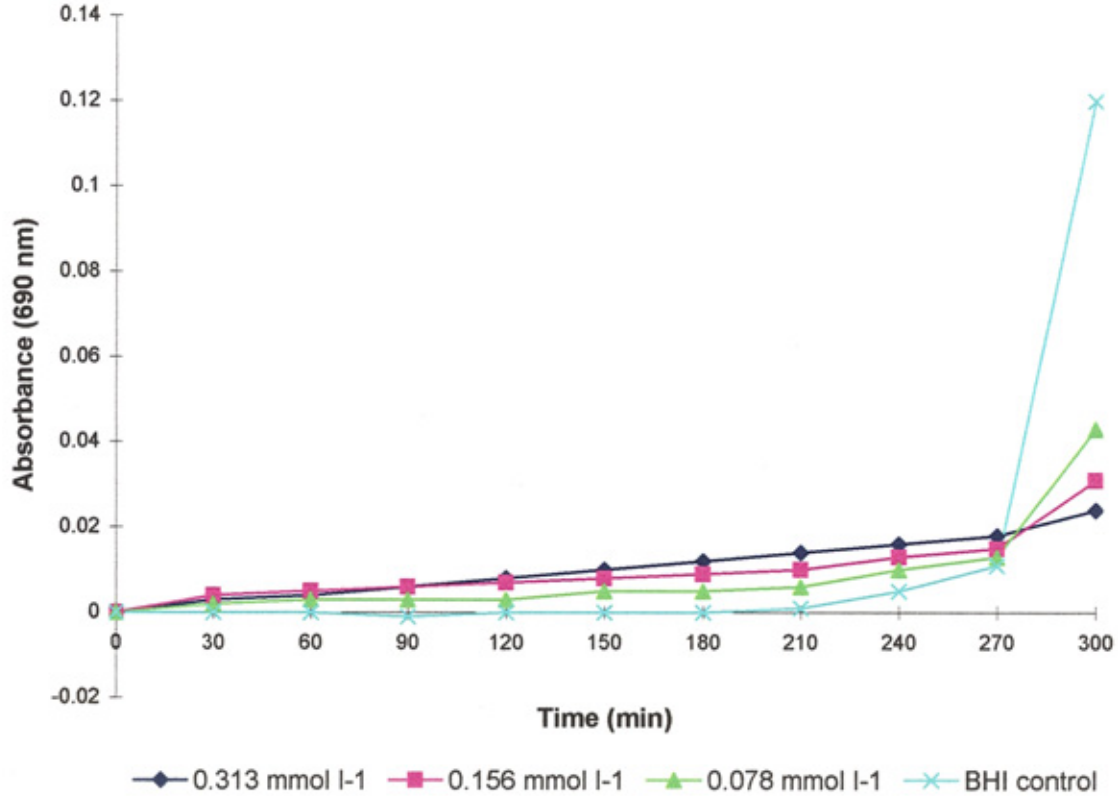


Figure 2.5.4: Growth of E.coli in the presence of various concentrations of diethyl-p-phenylenediamine.

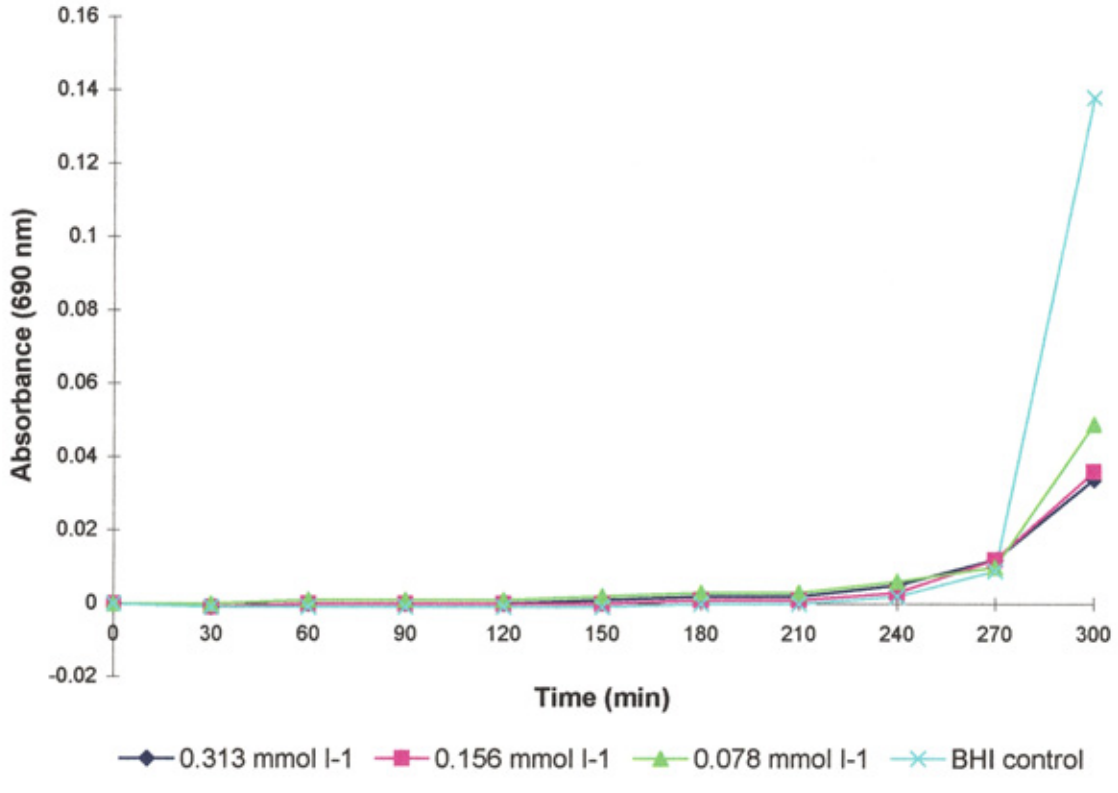


Figure 2.5.5: Growth of *E.coli* in the presence of various concentrations of dimethyl-*p*-phenylenediamine.

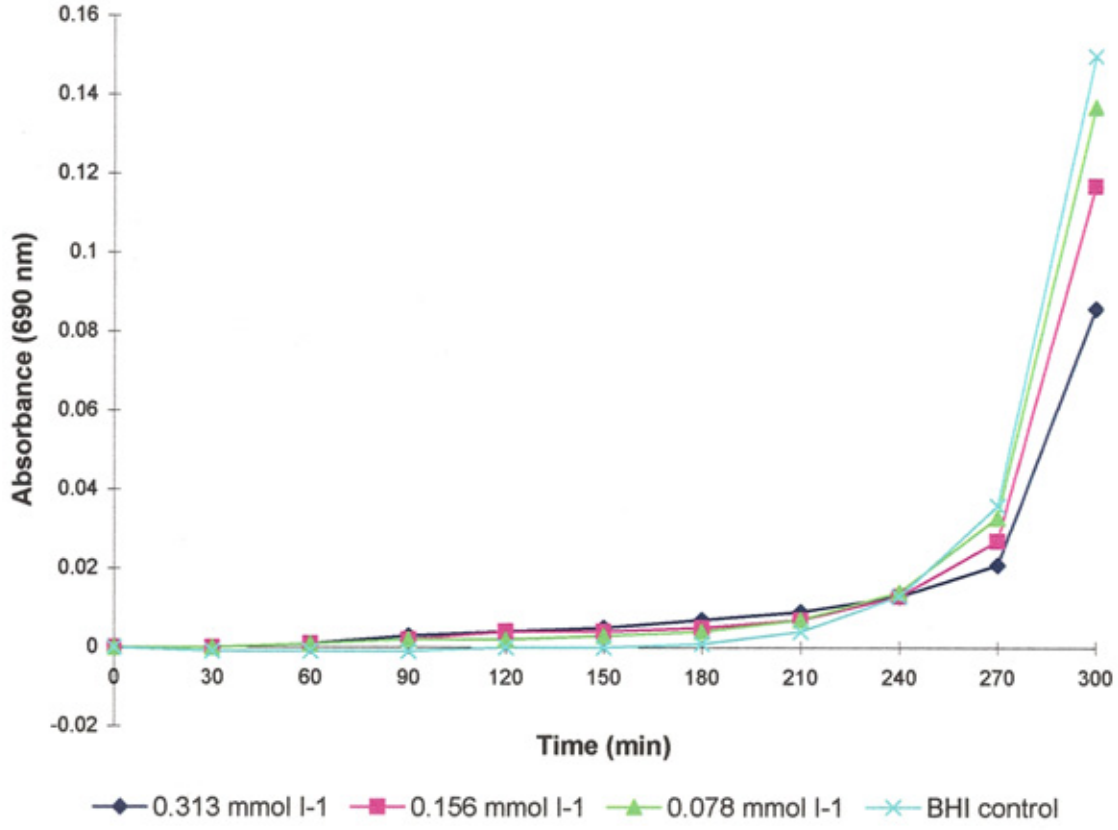


Figure 2.5.6. Growth of *S.marcescens* in the presence of various concentrations of 4-aminophenol

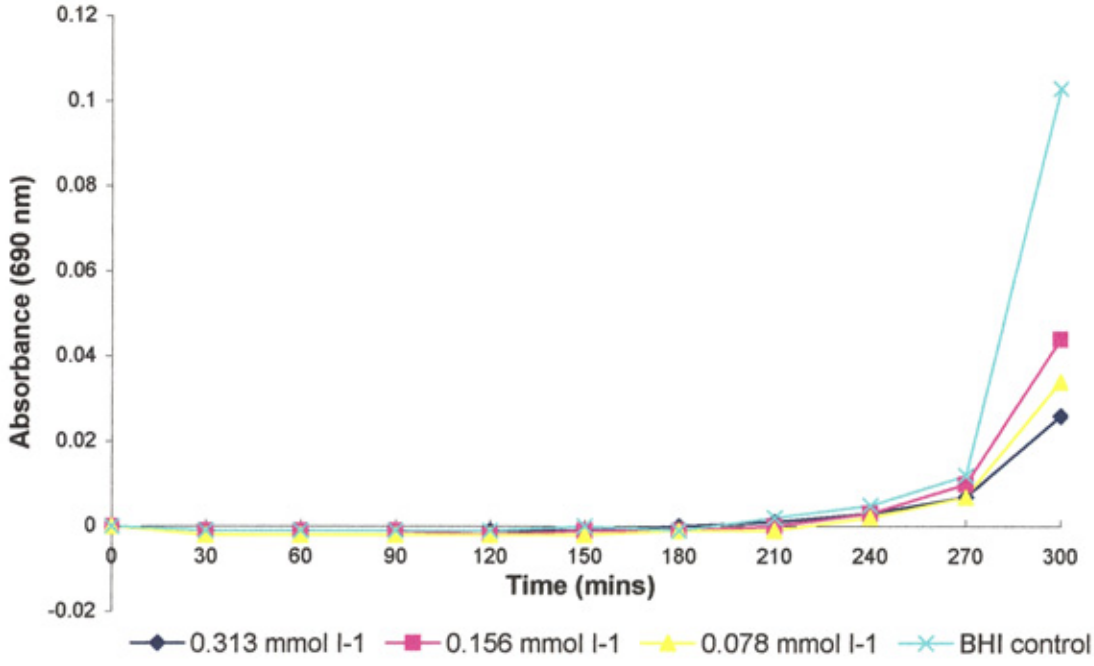


Figure 2.5.7. Growth of *S.aureus* in the presence of various concentrations of 4-aminophenol

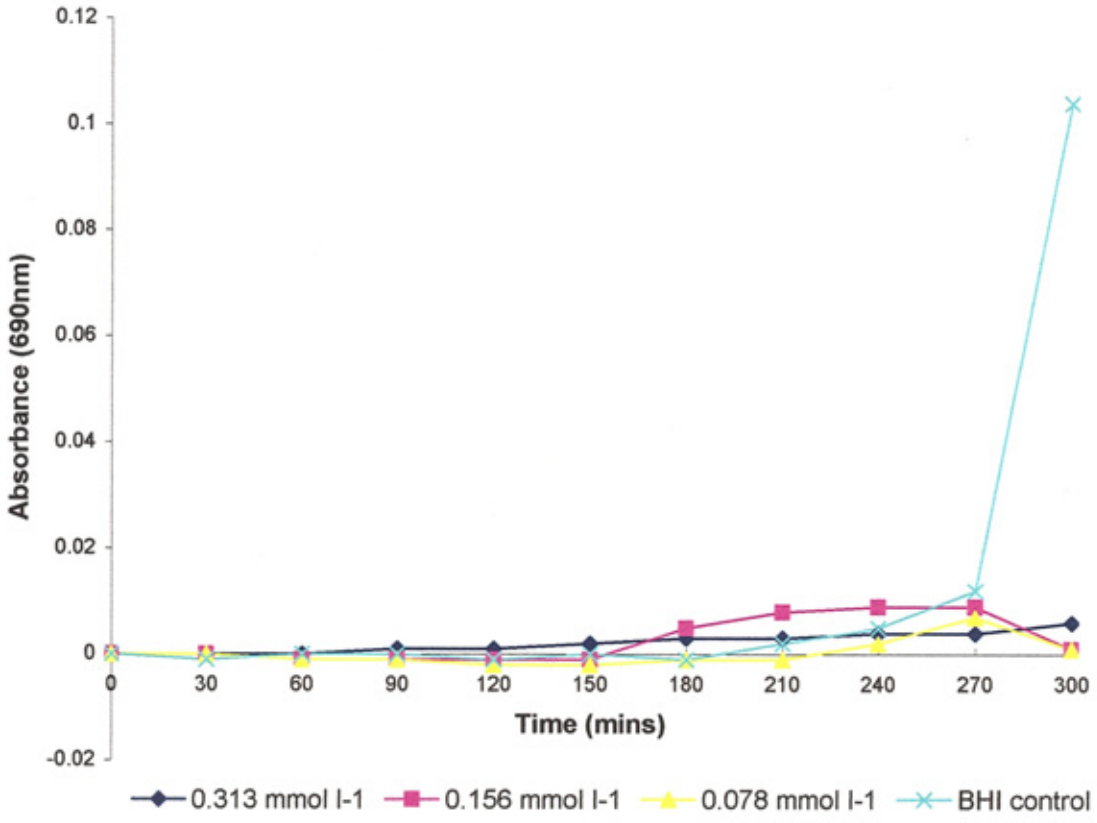
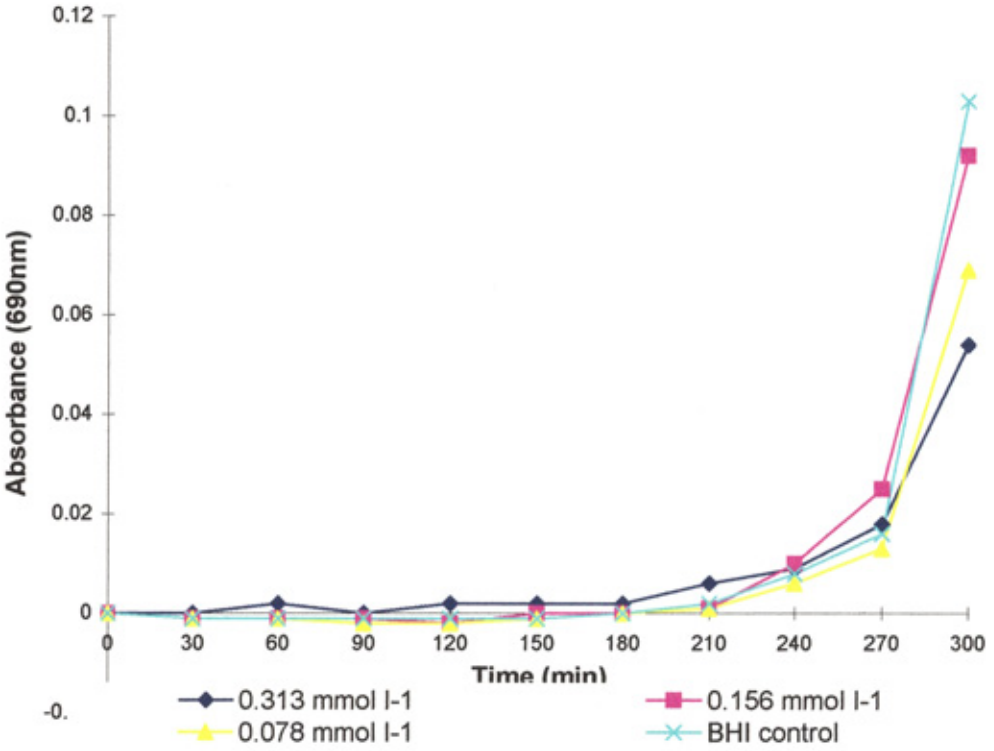


Figure 2.5.8. Growth of *E.faecalis* in the presence of various concentrations of 4-aminophenol



Determination of the toxicity of various naphthol derivatives against both Gram-positive and Gram-negative bacteria.

The complete results for this experiment are shown in Appendix 2.2, and are summarised in Table 2.1. The growth of *E.coli* (NCTC 10418) as measured by the increase in absorbance at 690 nm is shown in Figures 2.6.1 - 2.6.6. The BHI control well showed a rapid phase of growth after 120 minutes and continued for the remainder of the experiment. *E.coli* was completely inhibited by 1-naphthol (Fig 2.6.1) and 4-chloro-1-naphthol (Fig 2.6.3) at a concentration of 1 mmol l^{-1}. The former completely inhibited the growth of the organism at concentrations from 0.0625 mmol l^{-1} to 1 mmol l^{-1}. Interestingly, no toxicity was observed in the presence of 5[[(1-hydroxy-2-naphthyl)-carbonyl]octadecylamino]-isophthalic acid (Fig 2.6.5). Anthranol also demonstrated limited toxicity (Fig 2.6.2), as did 3,5-dihydroxy-2-naphthoic acid (Fig 2.6.4) and 3,5-dihydroxynaphthalene-2-carbonyl-2,4,6-trimentylanilide (Fig 2.6.6). For the other Gram-negative strains tested both 4-chloro-1-naphthol and 1-naphthol were highly toxic (Table 2.1). Moreover, limited toxicity was observed with both 3,5-dihydroxy-2-naphthoic acid and anthranol. Only at high concentrations (1 mmol l^{-1}) was any toxicity evident when the former compound was tested against Gram-negative bacteria. For the two Gram-positive strains examined, all of the naphthols showed a toxic effect. Growth of *S.aureus* (NCTC 6571) was inhibited by all naphthols, particularly 5[[(1-hydroxy-2-

naphthyl)-carbonyl]octadecylamino]-isophthalic acid (Fig 2.6.11), 3,5-dihydroxynaphthalene-2-carbonyl-2,4,6-trimentylanilide (Fig 2.6.12), 4-chloro-1-naphthol (Fig 2.6.9) and 1-naphthol (Fig 2.6.7). The napthol showing the least toxicity towards *S.aureus* was 3,5-dihydroxy-2-naphthoic acid (Fig 2.6.10). *E.faecalis* was also inhibited by these naphthols, and to a lesser extent by anthranol and 3,5-dihydroxy-2-naphthoic acid. Overall 1-naphthol and 4-chloro-1-naphthol had a significant inhibitory effect on all of the organisms tested. The least inhibitory compounds were 3,5-dihydroxy-2-naphthoic acid and anthranol (Table 2.1).

Table 2.1: Reduction in absorbance (690 nm) at 300 minutes for a range of naphthols and organisms. Figures are expressed as a percentage reduction compared to the growth control

	3,5-dihydroxy-2-naphthoic acid	5[[(1-hydroxy-2-naphthyl)-carbonyl]octadecylamino]-isophthalic acid	3,5-dihydroxynaphthalene-2-carbonyl-2,4,6-trimethylanilide	1-naphthol	anthranol	4-chloro-1-naphthol
S.aureus						
1 mmol l^{-1}	35	87	66	96	51	99
0.5 mmol l^{-1}	10	84	60	83	36	99
0.25 mmol l^{-1}	5	78	38	58	19	99
0.125 mmol l^{-1}	2	70	20	42	6	99
0.0625 mmol l^{-1}	0	52	10	20	4	52
E.faecalis						
1 mmol l^{-1}	42	100	88	88	28	100
0.5 mmol l^{-1}	27	100	56	70	24	100
0.25 mmol l^{-1}	5	97	35	45	10	98
0.125 mmol l^{-1}	8	93	16	28	2	76
0.0625 mmol l^{-1}	0	77	8	12	2	48
S.marcescens						
1 mmol l^{-1}	0	21	0	98	0	99
0.5 mmol l^{-1}	0	22	22	80	0	99
0.25 mmol l^{-1}	0	14	28	6	0	99
0.125 mmol l^{-1}	0	25	7	0	0	70
0.0625 mmol l^{-1}	0	23	0	0	6	0
S.typhimurium						
1 mmol l^{-1}	0	0	0	98	0	99
0.5 mmol l^{-1}	0	1	8	39	0	99
0.25 mmol l^{-1}	0	0	1	9	0	62
0.125 mmol l^{-1}	0	5	4	6	0	17
0.0625 mmol l^{-1}	0	5	0	3	0	9
E.cloacae						
1 mmol l^{-1}	22	3	37	99	6	99
0.5 mmol l^{-1}	6	0	31	68	0	99
0.25 mmol l^{-1}	1	5	18	35	2	75
0.125 mmol l^{-1}	0	3	5	21	0	12
0.0625 mmol l^{-1}	0	3	5	15	2	6
K.pneumoniae						
1 mmol l^{-1}	16	28	49	100	0	99
0.5 mmol l^{-1}	0	33	52	77	0	99
0.25 mmol l^{-1}	0	30	48	57	0	82
0.125 mmol l^{-1}	0	33	10	34	0	32
0.0625 mmol l^{-1}	0	28	0	11	0	12
E.coli						
1 mmol l^{-1}	14	0	65	100	11	100
0.5 mmol l^{-1}	25	0	56	78	5	100
0.25 mmol l^{-1}	20	0	35	38	0	100
0.125 mmol l^{-1}	8	1	18	17	0	100
0.0625 mmol l^{-1}	0	0	8	8	2	69

Figure 2.6.1 : Growth of E.coli in the presence of various concentrations of 1-naphthol.

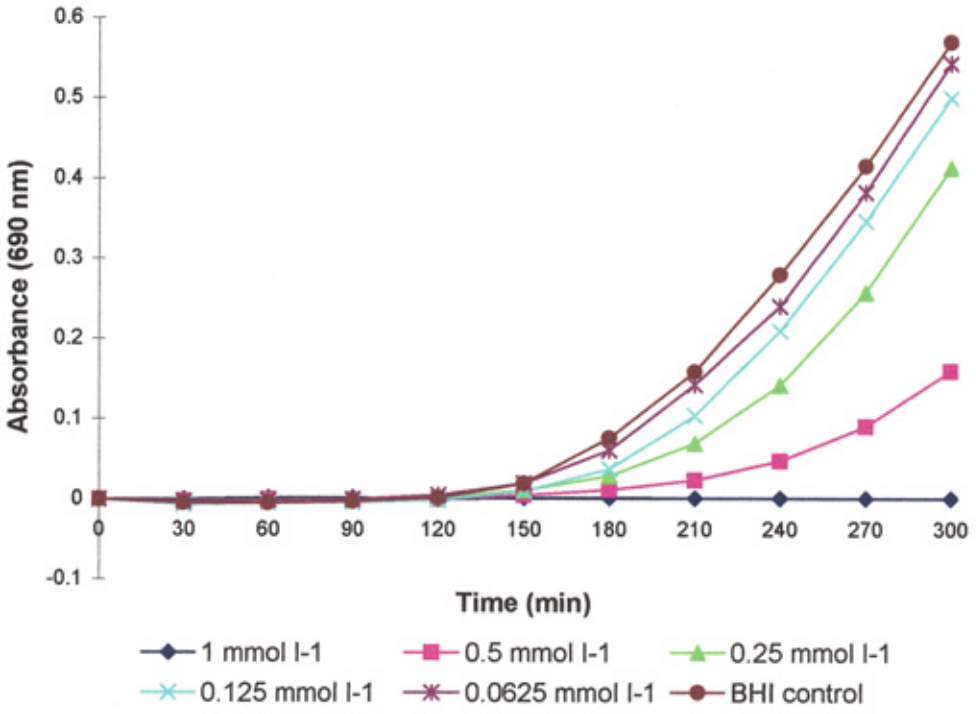


Figure 2.6.2: Growth of E.coli in the presence of various concentrations of anthranol.

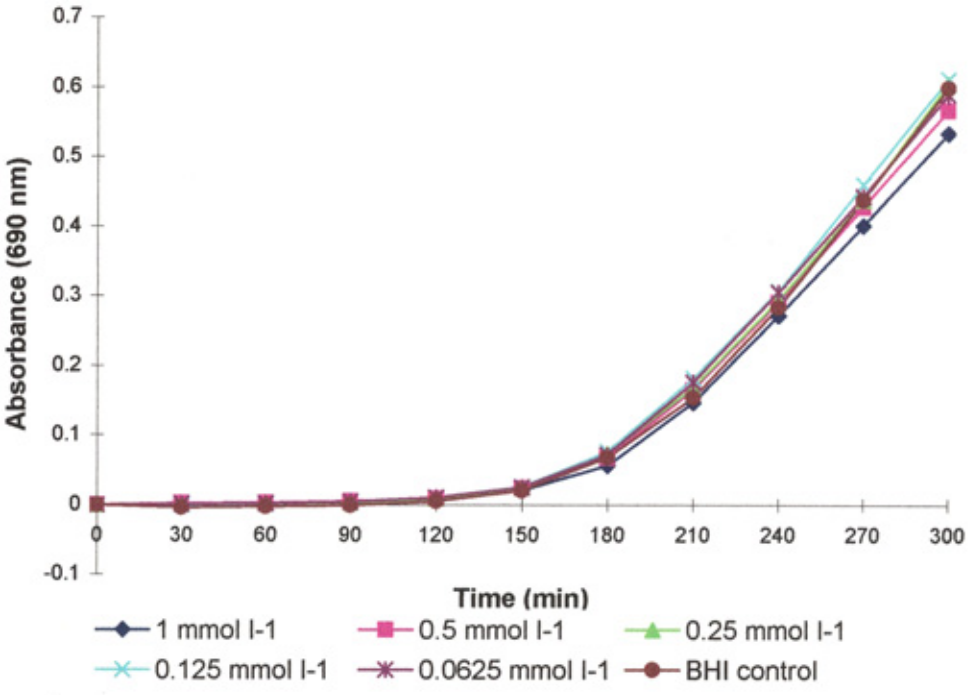


Figure 2.6.3: Growth of *E.coli* in the presence of various concentrations of 4-chloro-1-naphthol.

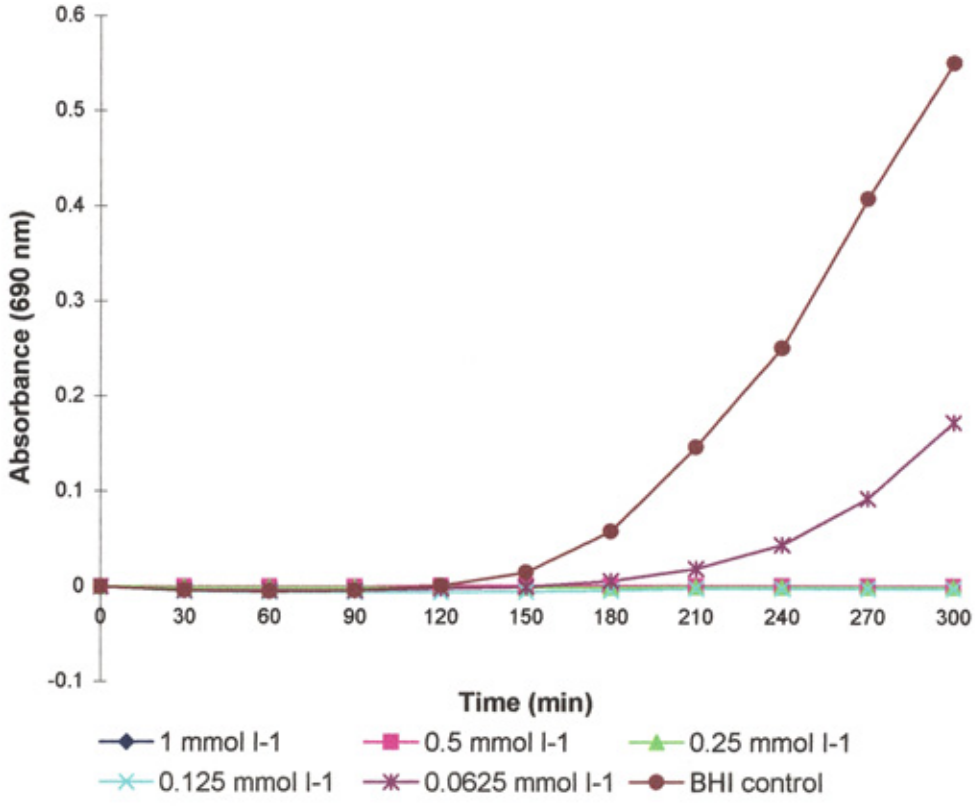


Figure 2.6.4: Growth of *E.coli* in the presence of various concentrations of 3,5-dihydroxy-2-naphthoic acid.

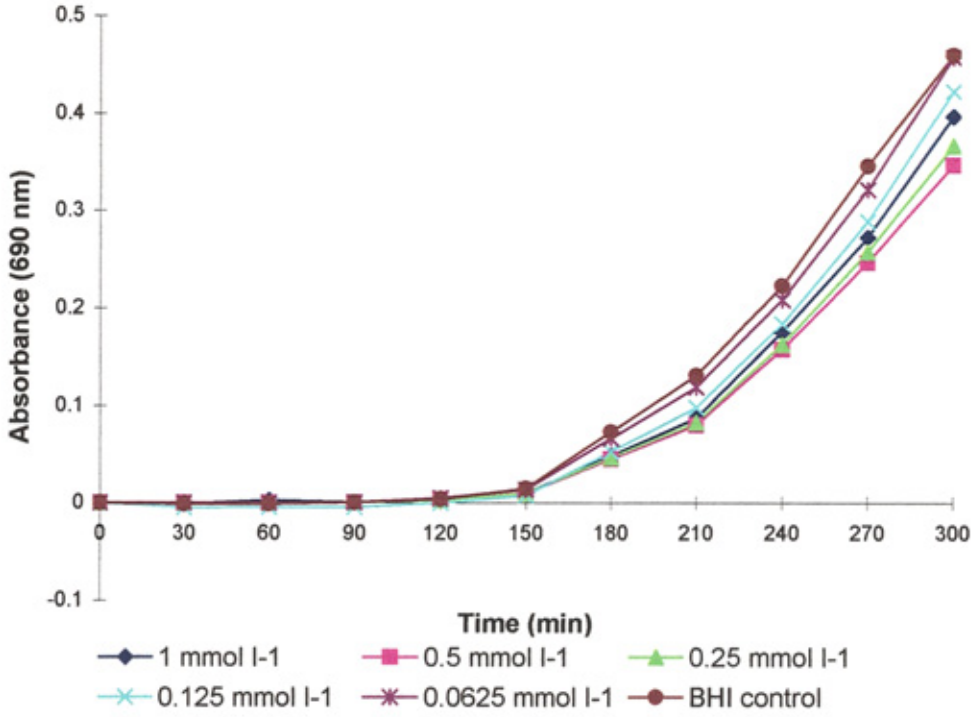


Figure 2.6.5: Growth of E.coli in the presence of various concentrations of 5[[(1-hydroxy-2-naphthyl)-carbonyl]octadecylamino]-isophthalic acid.

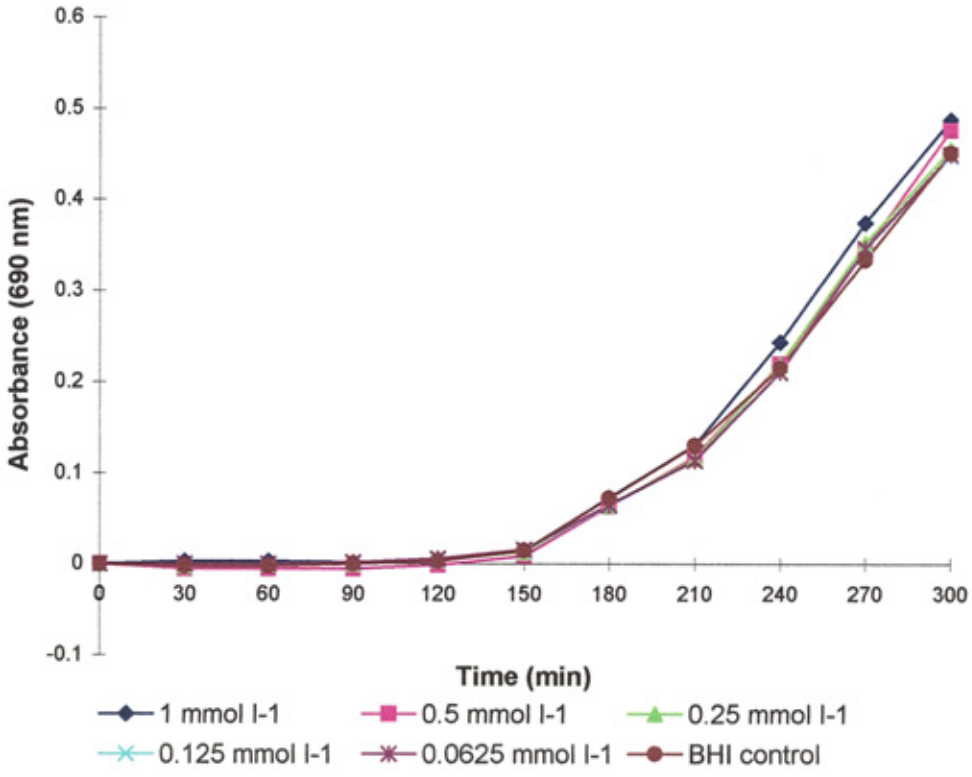


Figure 2.6.6: Growth of E.coli in the presence of various concentrations of 3,5-dihydroxynaphthalene-2-carbonyl-2,4,6-trimethylanilide

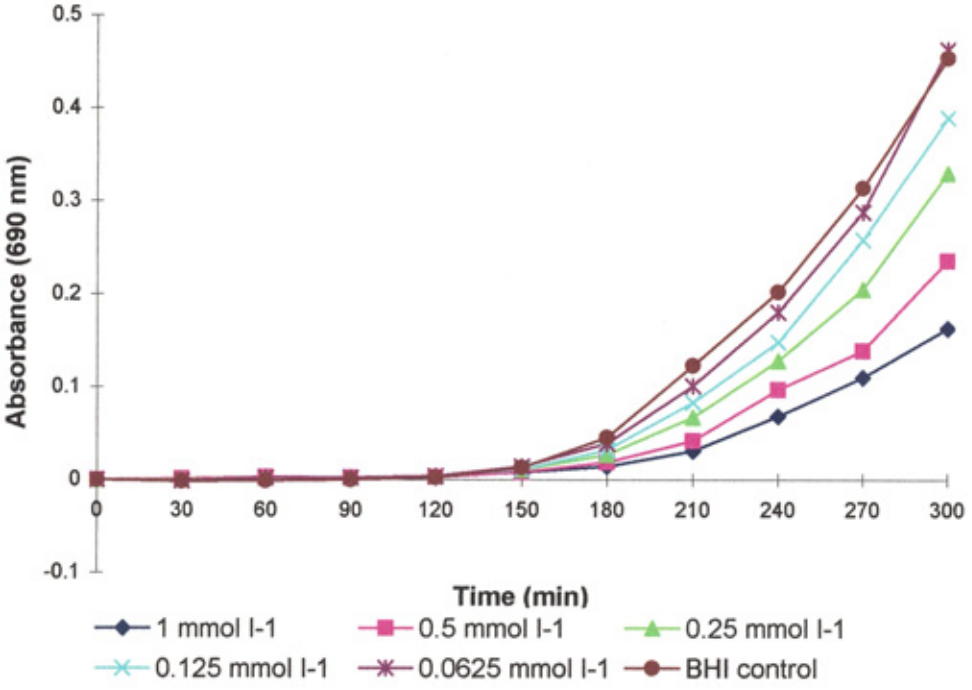


Figure 2.6.7: Growth of *S.aureus* in the presence of various concentrations of 1-naphthol.

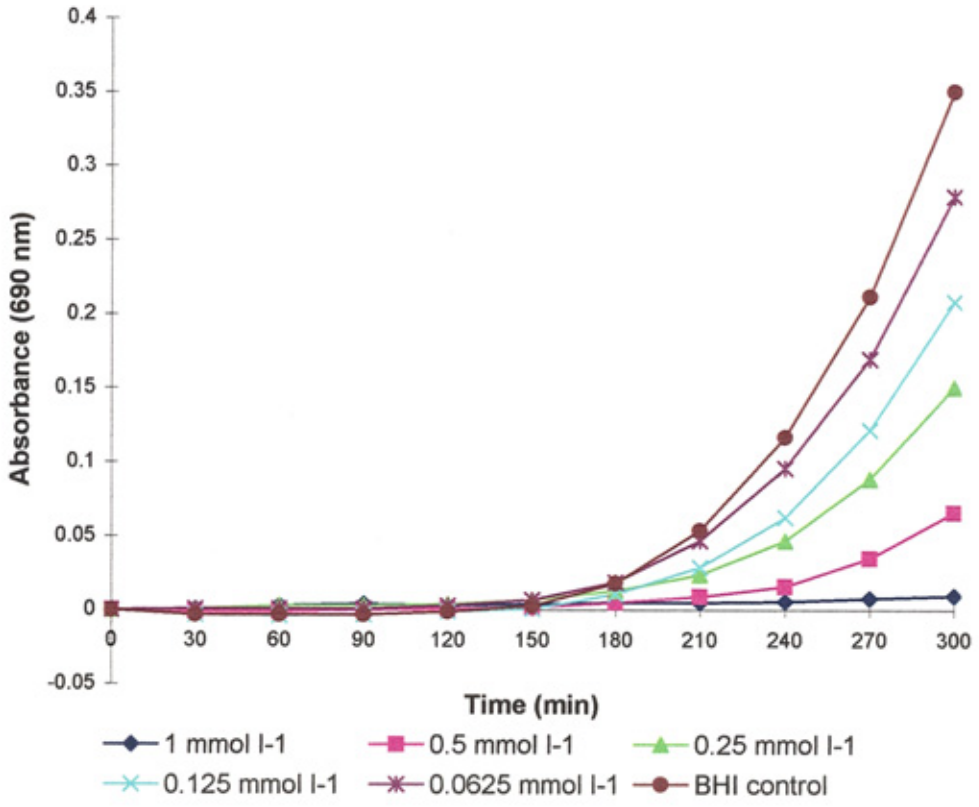


Figure 2.6.8: Growth of *S.aureus* in the presence of various concentrations of anthranol.

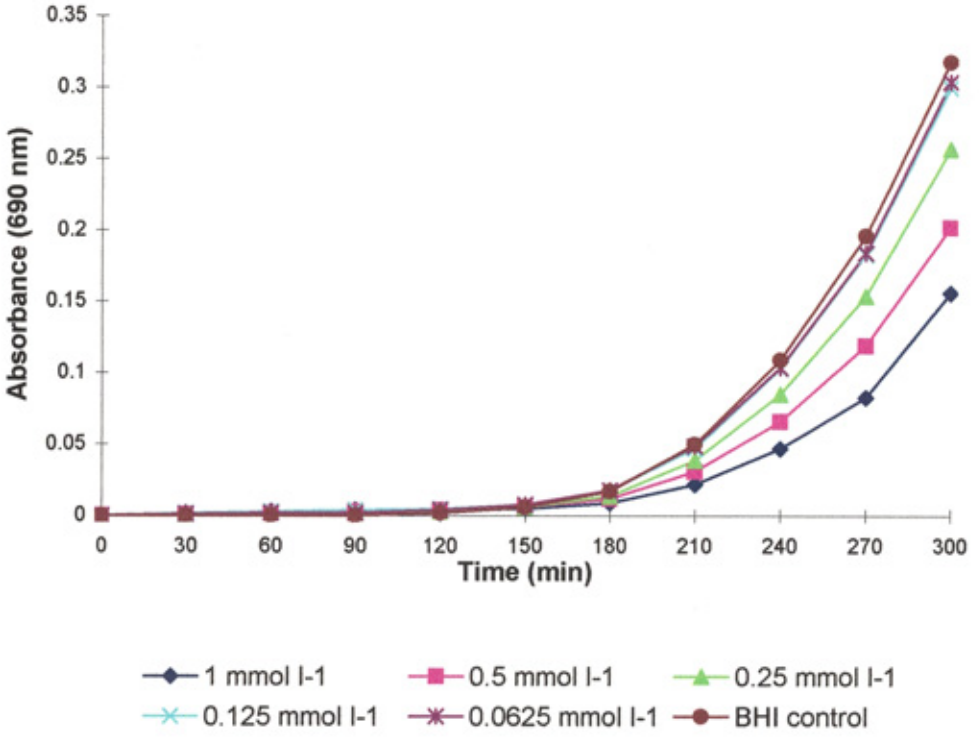


Figure 2.6.9: Growth of S.aureus in the presence of various concentrations of 4-chloro-1-naphthol.

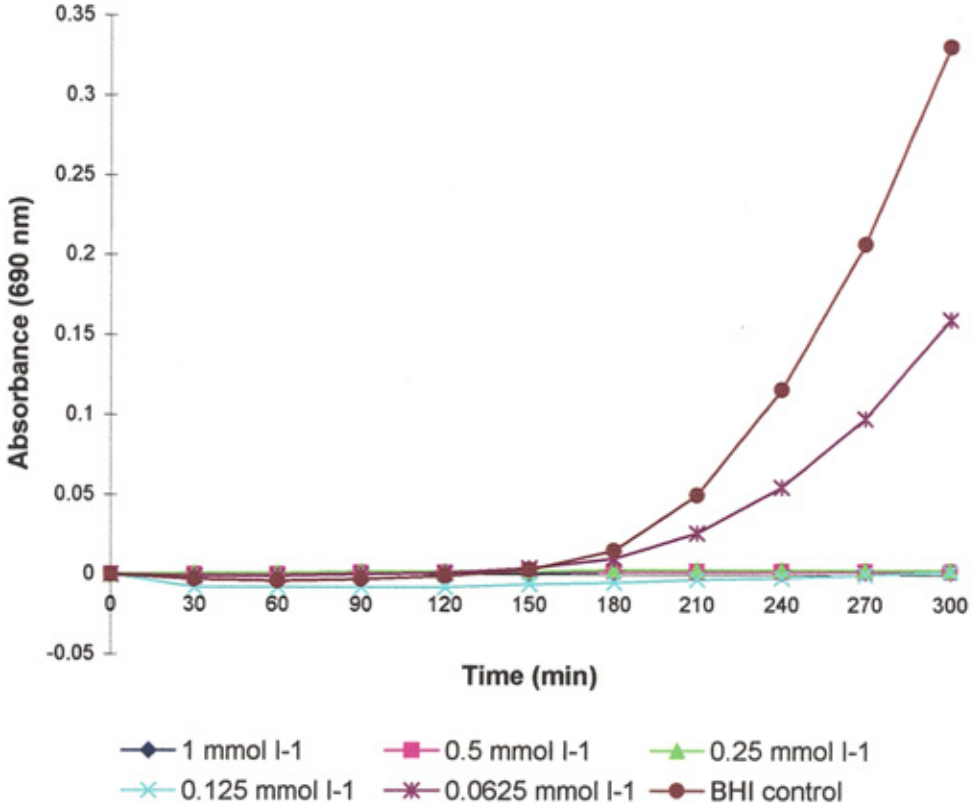


Figure 2.6.10: Growth of S.aureus in the presence of various concentrations of 3,5-dihydroxy-2-naphthoic acid.

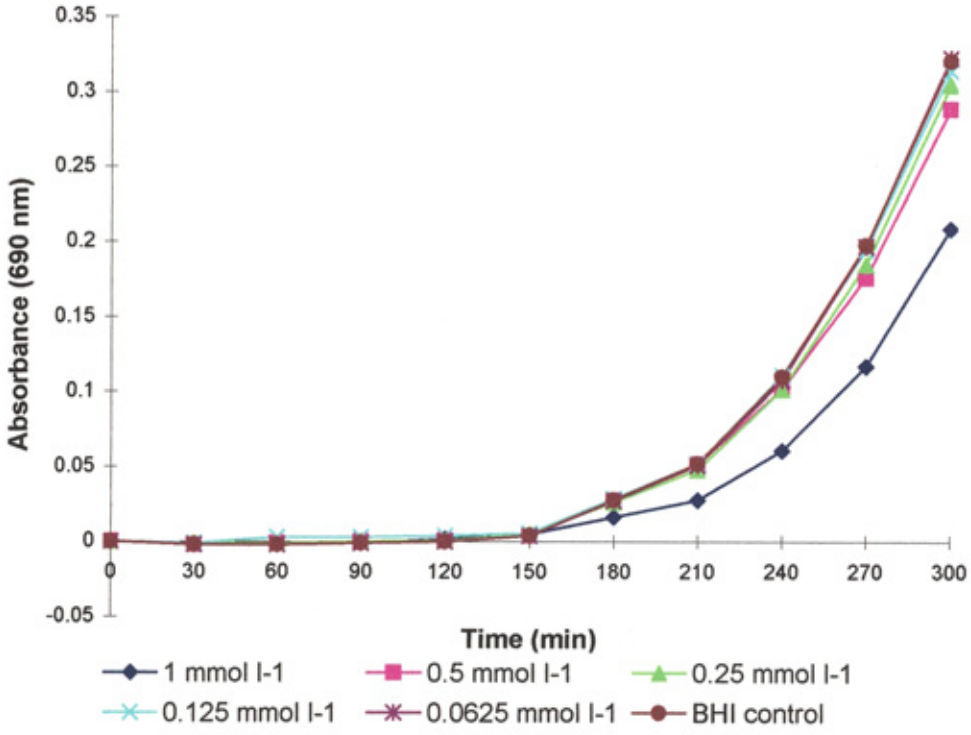


Figure 2.6.11: Growth of *S.aureus* in the presence of various concentrations of 5[[(1-hydroxy-2-naphthyl)-carbonyl]octadecylamino]-isophthalic acid.

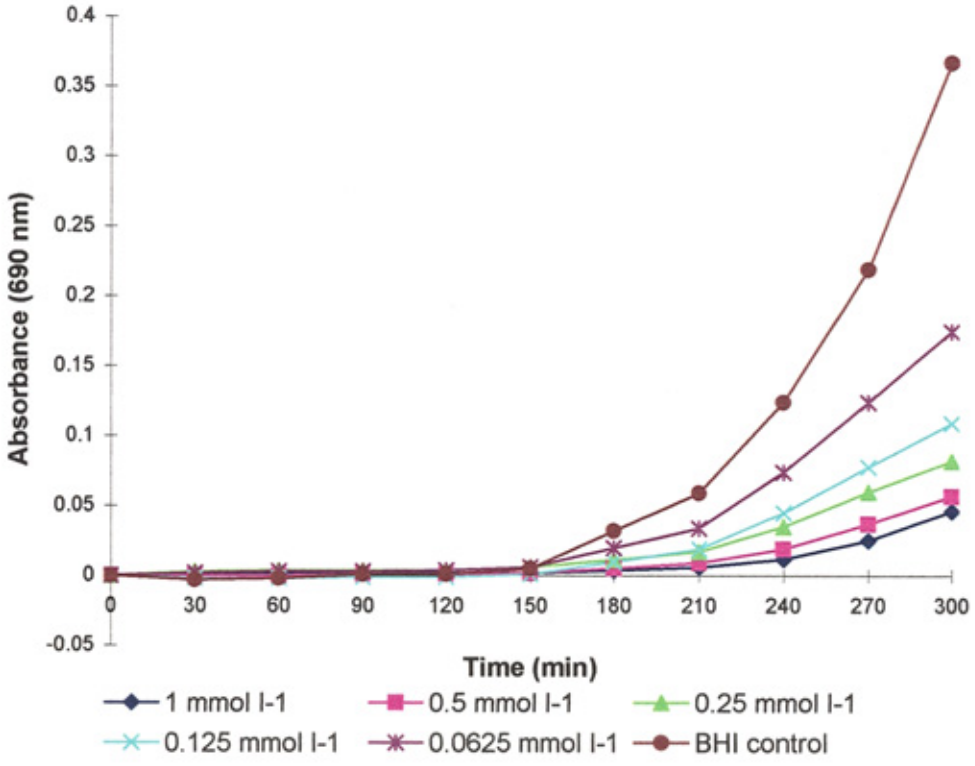
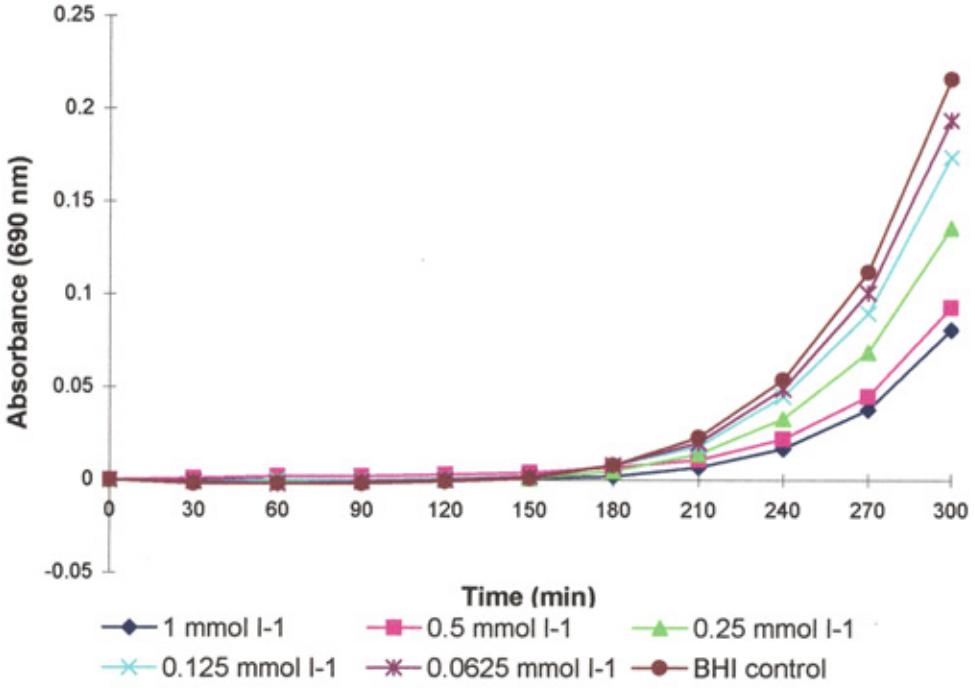


Figure 2.6.12: Growth of *S.aureus* in the presence of various concentrations of 3,5-dihydroxynaphthalene-2-carbonyl-2,4,6-trimethylanilide



The relative growth inhibition by many of the test naphthols may not be as relevant when testing for pre-formed enzymes using a heavy inoculum, since the intensity of the coloured reaction product will be priority. However the toxicity data are relevant in relation to the incorporation of such derivatives into agar-based growth media.

Determination of the toxicity of various L-alanyl substrates against both Gram-negative and Gram-negative bacteria.

The full data for this experiment are displayed in Appendix 2.3. The growth of *E.coli* in the presence of all L-alanyl substrates is shown in figures 2.7.1 to 2.7.5. From the figures the growth of *E.coli* was only slightly inhibited by L-alanyl-DEPPD (Fig 2.7.2) but not by any of the other L-alanyl substrates tested. Of the other Gram-negative strains examined no significant growth inhibition was observed with L-alanyl-4-aminophenol, or the halogenated substrates, except for *K.pneumoniae* which was partially inhibited after 300 minutes in the presence of 0.625 mmol l^{-1} L-alanyl-4-amino-2,6-dibromophenol (Fig 2.7.11). Of the Gram-positive strains, the growth of *S.aureus*, was inhibited significantly by L-alanyl derivatives of 4-amino-2,6-dichlorophenol (Fig 2.7.10), 4-amino-2,6-dibromophenol (Fig 2.7.9) and DEPPD (Fig 2.7.6). L-alanyl derivatives of 4-aminophenol (Fig 2.7.8) and DMPPD (Fig 2.7.7) appeared to be relatively non-inhibitory to *S.aureus*. Growth of *E.faecalis* was partially inhibited by both *p*-phenylenediamines

and all aminophenol substrates. Most growth inhibition was observed with this strain in the presence of L-alanyl-4-amino-2,6-dichlorophenol.

Figure 2.7.1: Growth of *E.coli* in the presence of various concentrations of L-alanyl-dimethyl-*p*-phenylenediamine.

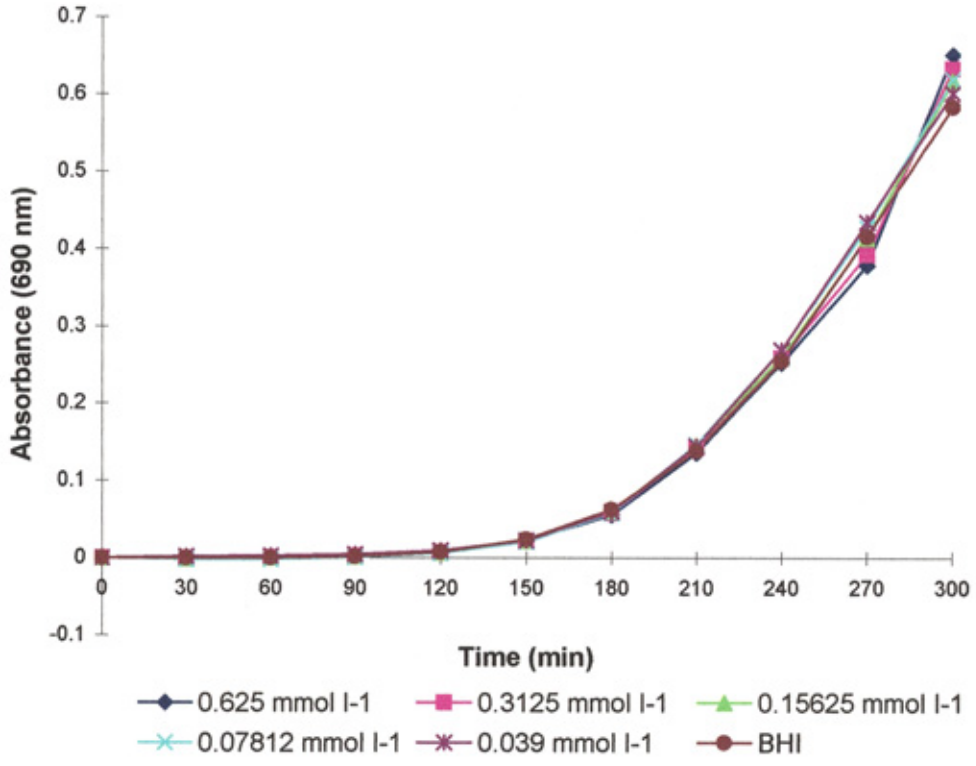


Figure 2.7.2: Growth of *E.coli* in the presence of various concentrations of L-alanyl-diethyl-*p*-phenylenediamine.

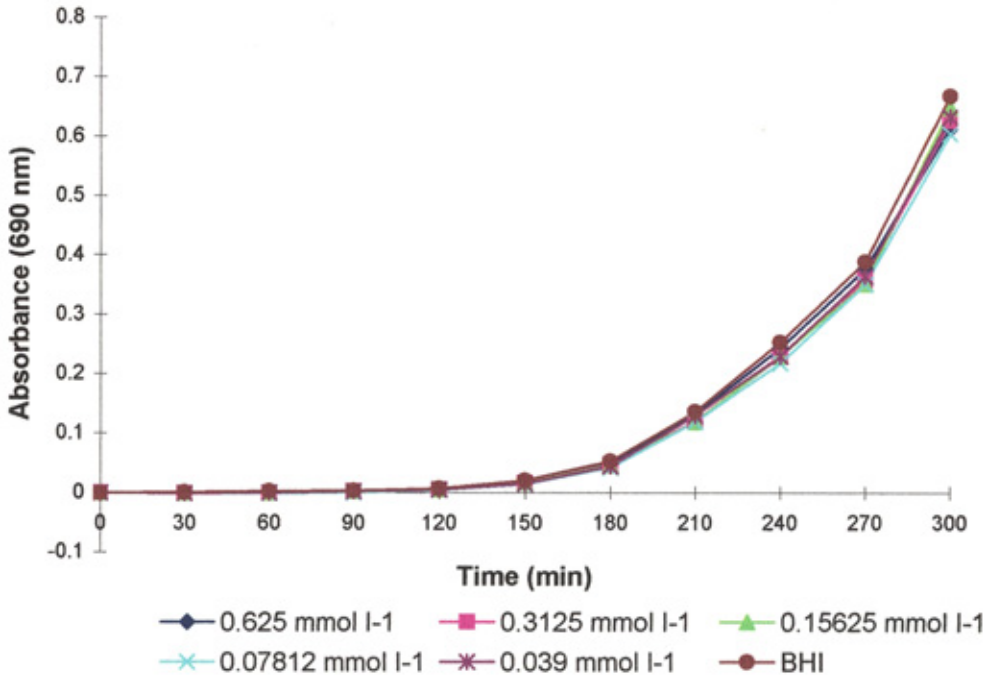


Figure 2.7.3: Growth of *E.coli* in the presence of various concentrations of L-alanyl-4-aminophenol.

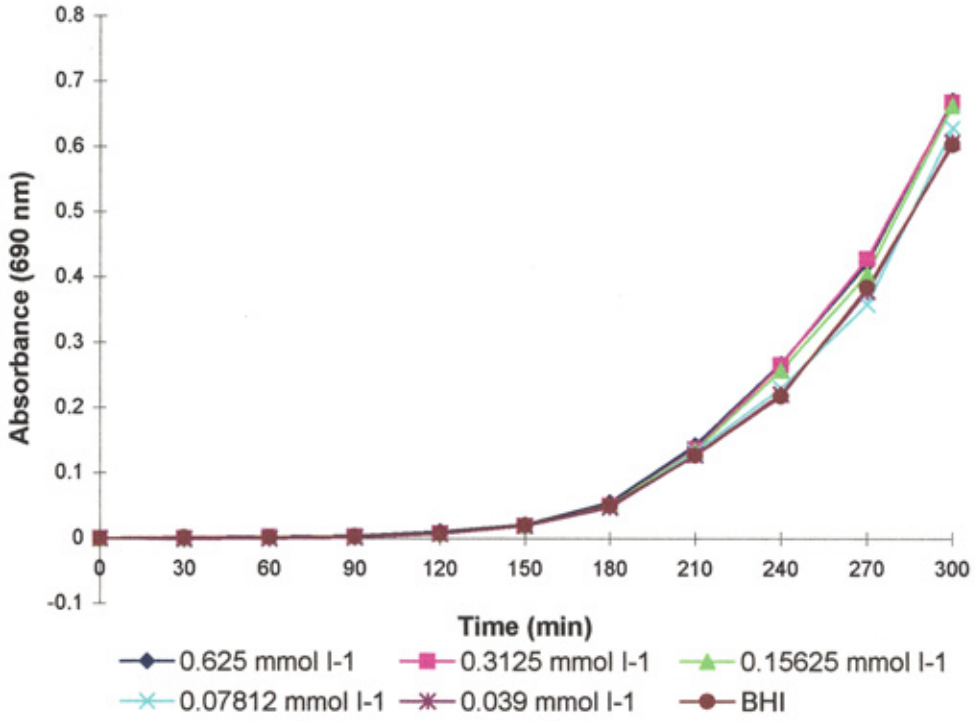


Figure 2.7.4: Growth of *E.coli* in the presence of various concentrations of L-alanyl-4-amino-2,6-dibromophenol.

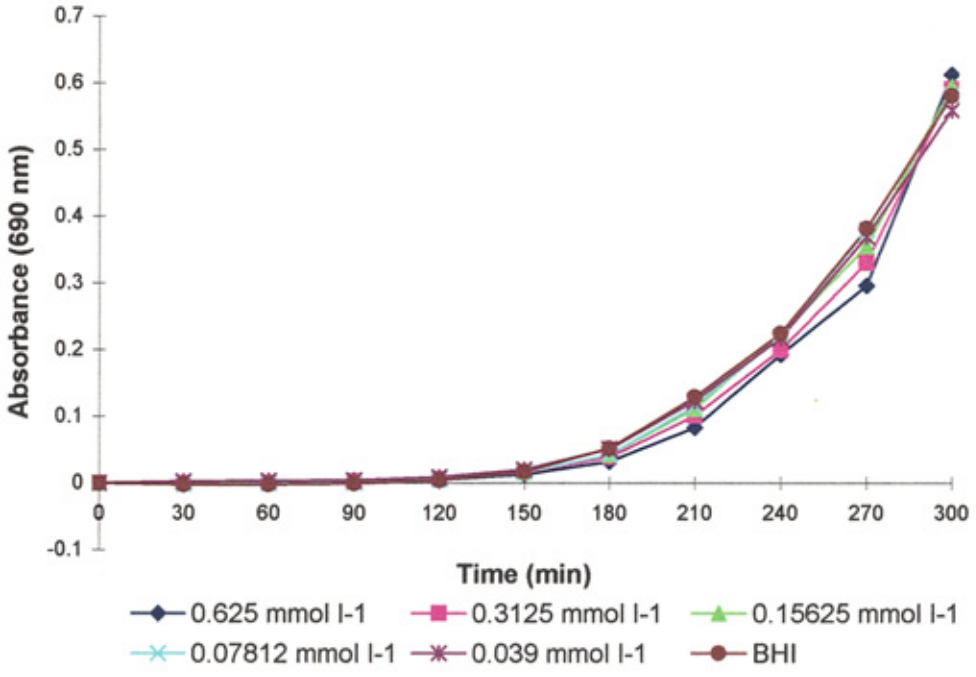


Figure 2.7.5: Growth of E.coli in the presence of various concentrations of L-alanyl-4-amino-2,6-dichlorophenol.

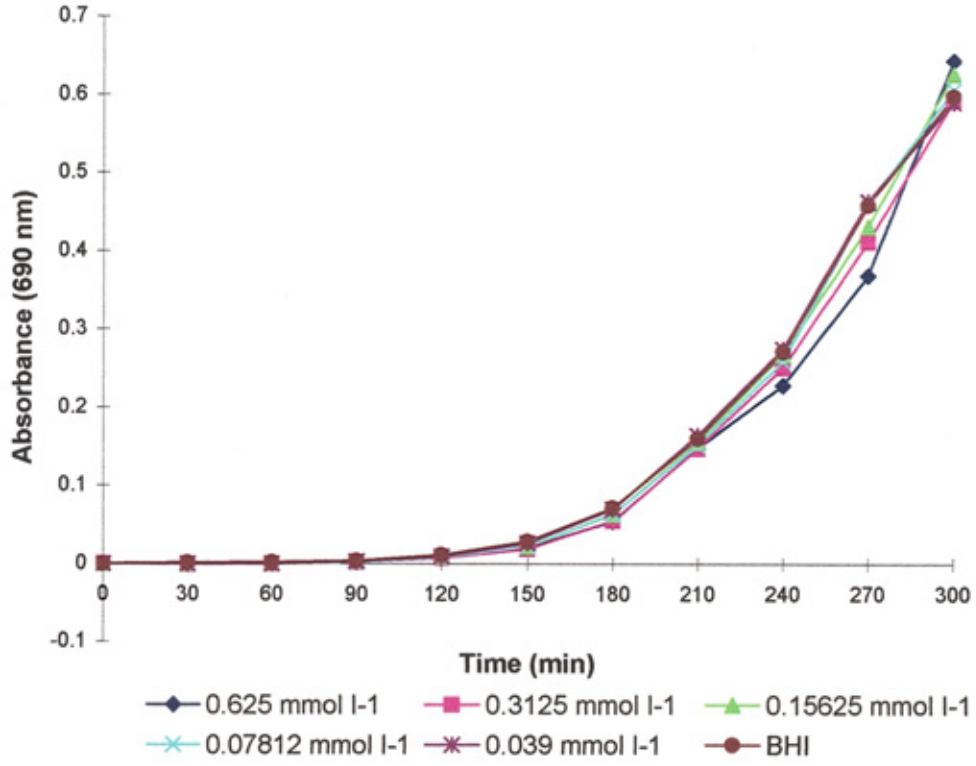


Figure 2.7.6. Growth of S.aureus in the presence of various concentrations of L-alanyl-diethyl-p-phenylenediamine.

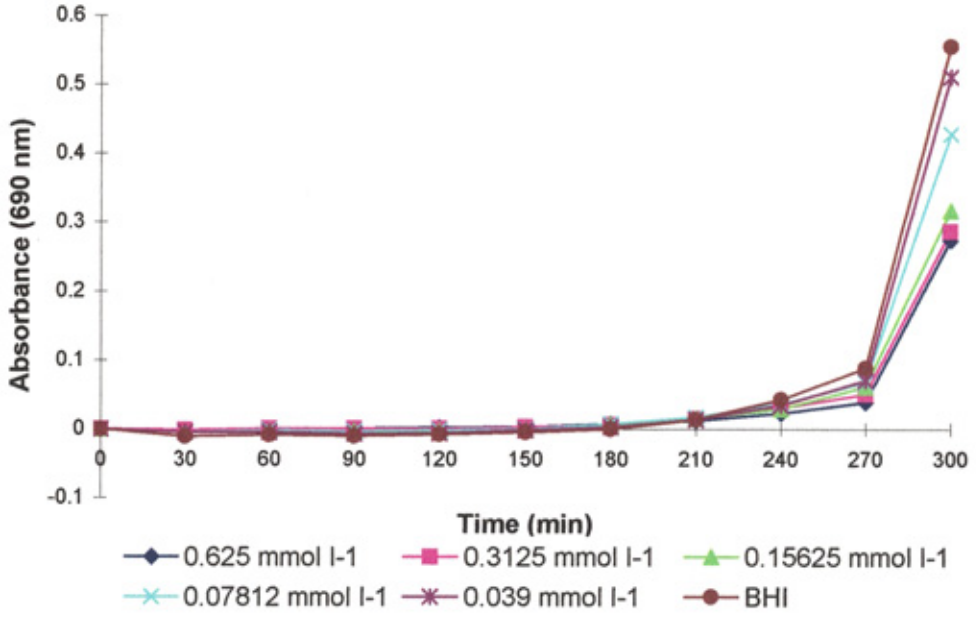


Figure 2.7.7.: Growth of *S.aureus* in the presence of various concentrations of L-alanyl-dimethyl-*p*-phenylenediamine.

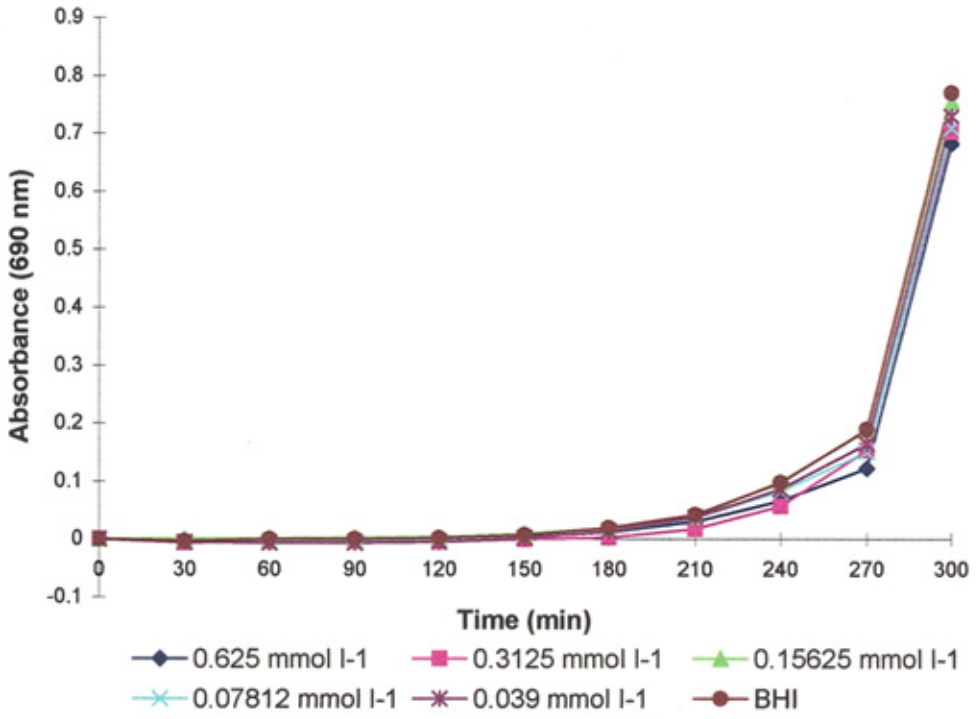


Figure 2.7.8: Growth of *S.aureus* in the presence of various concentrations of L-alanyl-4-aminophenol.

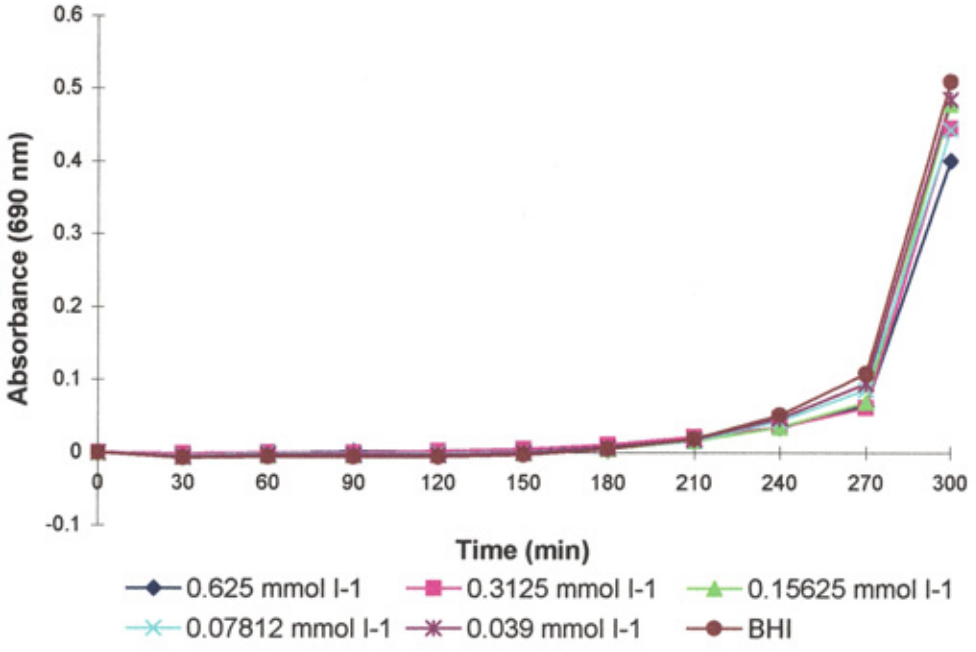


Figure 2.7.9: Growth of *S.aureus* in the presence of various concentrations of L-alanyl-4-amino-2,6-dibromophenol.

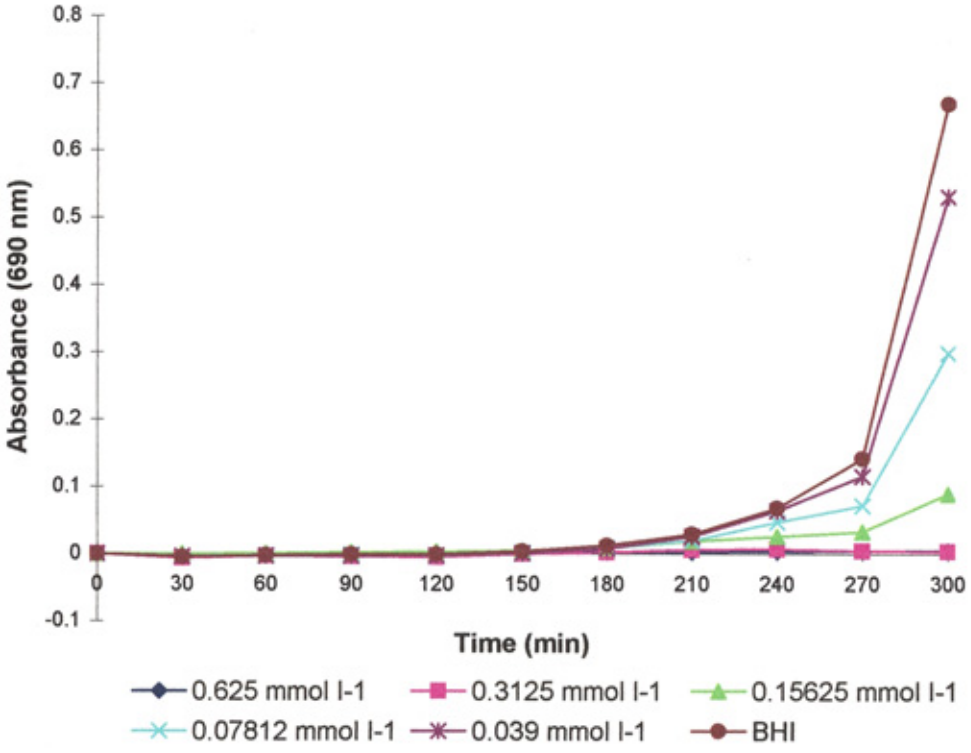


Figure 2.7.10: Growth of *S.aureus* in the presence of various concentrations of L-alanyl-4-amino-2,6-dichlorophenol.

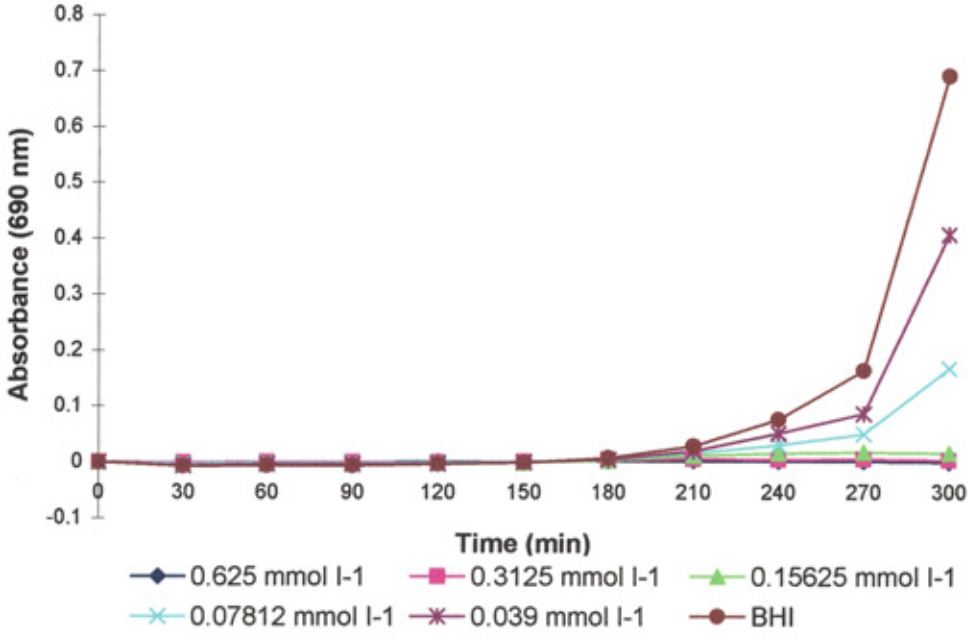
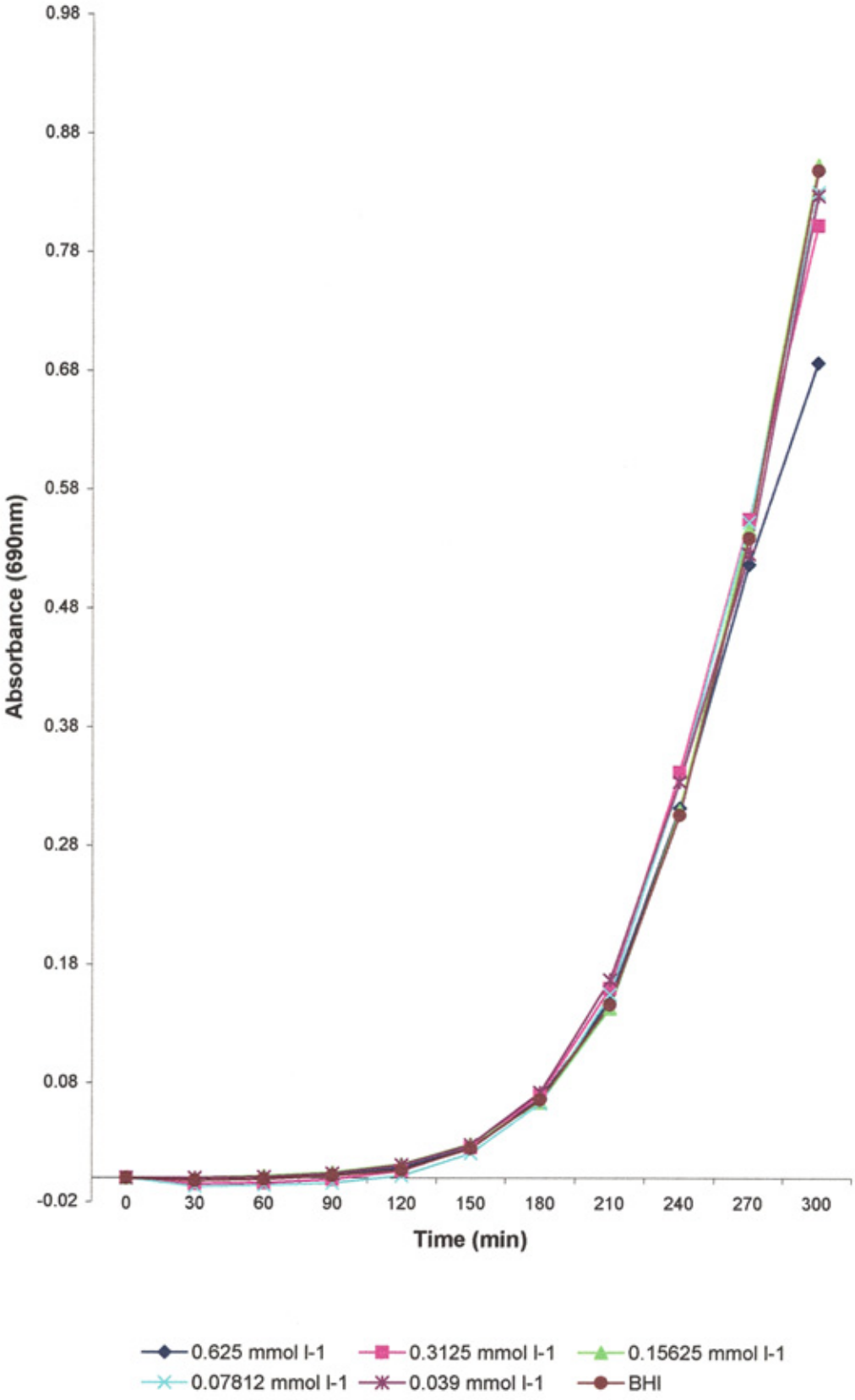


Figure 2.7.11: Growth of *K.pneumoniae* in the presence of various concentrations of L-alanyl-4-amino-2,6-dibromophenol



Determination of the optimal conditions for colour development produced by *E.coli* in the presence of L-alanyl-DEPPD and 3,5-dihydroxy-2 naphthoic acid.

The full data for this experiment are displayed in Appendix 2.4. It was clear from the results that the most intense colour production occurred in wells in which there was a concentration of at least 1.25 mmol l^{-1} of 3,5-dihydroxy-2-naphthoic acid accompanied by a concentration of at least 1.25 mmol l^{-1} L-alanyl-DEPPD. It was also clear that the higher the inoculum of *E.coli*, the more intense the reaction. Figure 2.8 shows the effect of volume on the formation of the indophenol complex, the highest amount of indamine produced as judged by difference in absorbance between 690 and 405 nm was when the volume was 200 µl. At 100 µl and 50 µl the intensity of the coloured product formed was much less. There was little difference between a volume of 100 µl and 50 µl. Figure 2.9 shows the effect of inoculum of *E.coli* in the formation of the indophenol complex. The higher the inoculum the more indamine blue was generated over a four hour incubation period with the maximum colour observed at an inoculum of $1x10^9$ cfu ml^{-1}. At the two other inocula examined, colour was observed, but the colour observed was much weaker at an inoculum of $4x10^8$ cfu ml^{-1}. This would suggest that for studies in liquid media an inoculum of around 10^9cfu ml^{-1} is required for optimal production of the blue indophenol complex. This would have some

impact on routine diagnostic use, since several individual bacterial colonies might be required to reach the required inoculum, depending on colony size.

Determination of the optimal conditions for colour development produced by a range of organisms in the presence of L-alanyl-DEPPD and 3,5-dihydroxy-2-naphthoic acid.

The complete data for this experiment are shown in Appendix 2.5, which shows that the optimal concentration of substrate was 2.5 mmol l^{-1} for the range of organisms tested. Different species however differed in their optimal concentration of 3,5-dihydroxy-2-naphthoic acid. For example, Figure 2.10.1 shows that *E.coli* required 1.25 mmol l^{-1} of 3,5-dihydroxy-2-naphthoic acid for optimal production of the indophenol blue complex. Below this concentration however, colour production was still evident visibly, even at the lowest concentration of naphthol used (0.156 mmol l^{-1}). In contrast, 0.3125 mmol l^{-1} of 3,5-dihydroxy-2-naphthoic acid was optimal for *K.pneumoniae* (Appendix 2.5) whereas *P.aeruginosa* required the lowest concentration of the naphthol at 0.156 mmol l^{-1} (Fig. 2.10.2). It was interesting to note that for *P.aeruginosa* the highest naphthol concentration tested (5 mmol l^{-1}) produced the least amount of coloured reaction product. The reasons for this may be related to the toxicity of the naphthol for this organism. For the other Gram-negative strains tested, *S.typhimurium* and *E.cloacae,* the optimal naphthol concentrations were 2.5 and 1.25 mmol l^{-1}

respectively (Appendix 2.5). As expected very little colour was formed in the wells containing the Gram-positive strains *S.aureus* and *E.faecalis* (Fig 2.10.3) since these organisms lack L-alanyl-aminopeptidase. It was decided that a suitable concentration of 3,5-dihydroxy-2-naphthoic acid for colour development in the presence of L-alanyl-DEPPD was 0.625 mmol l^{-1} as this was closest to the average optimal concentration as shown in Fig.2.10.4.

Figure 2.8: Production of indamine complex by *E.coli* at various volumes L-alanyl-DEPPD (1.25 mmol l^{-1}) in the presence of 1-naphthol (1.25 mmol l^{-1}

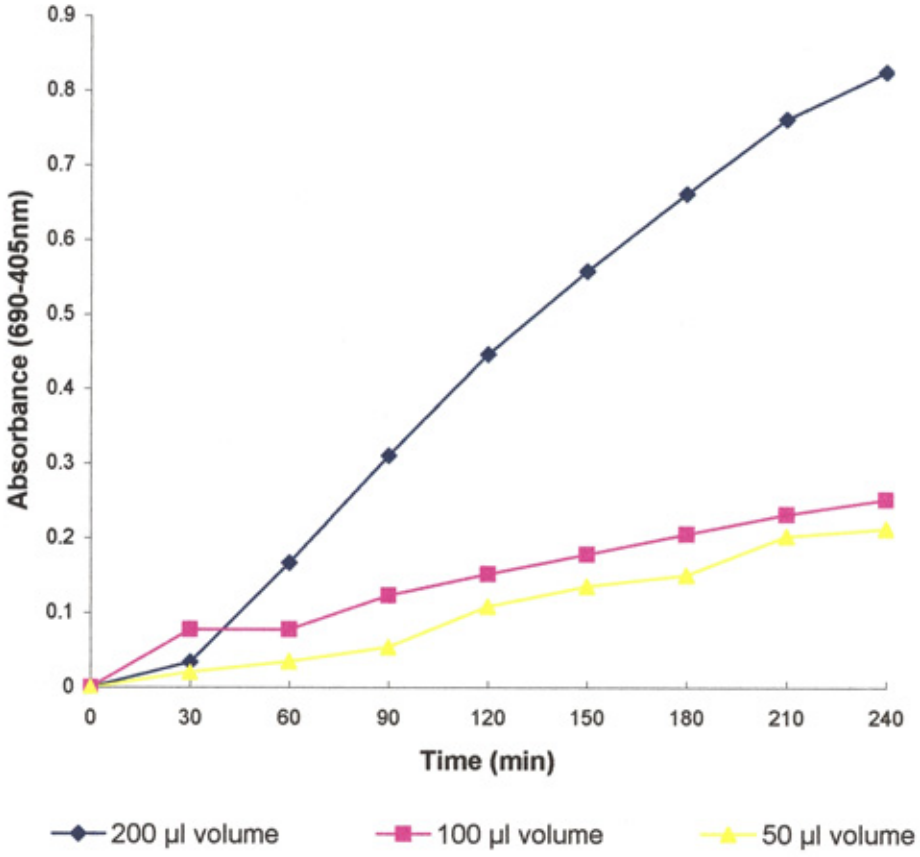


Figure 2.9: Production of indamine complex by *E.coli* at various volumes due to hydrolysis of L-alanyl-DEPPD 1.25 mmol l^{-1} in the presence of 1-naphthol 1.25 mmol l^{-1}.

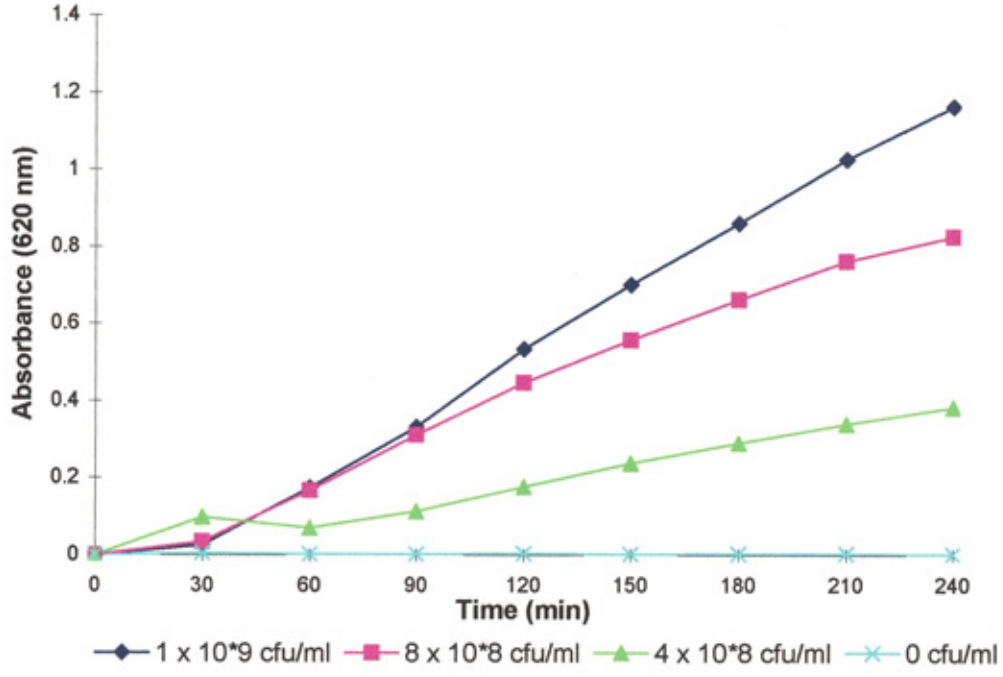


Figure 2.10.1: Generation of indamine complex by *E.coli* due to hydrolysis of L-alanyl-DEPPD (2.5 mmol l^{-1}) in the presence of varying concentrations of 3,5-dihydroxy-2-naphthoic acid

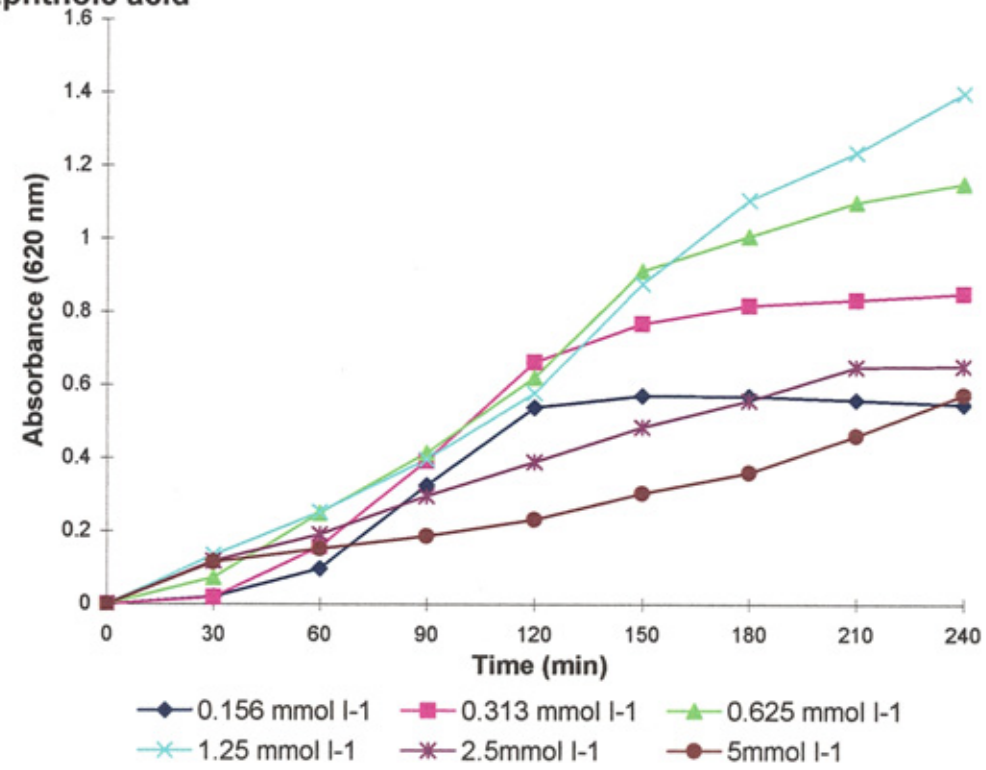


Figure 2.10.2: Generation of indamine complex by *P.aeruginosa* due to hydrolysis of L-alanyl-DEPPD (2.5 mmol l^{-1}) in the presence of varying concentrations of 3,5-dihydroxy-2-naphthoic acid.

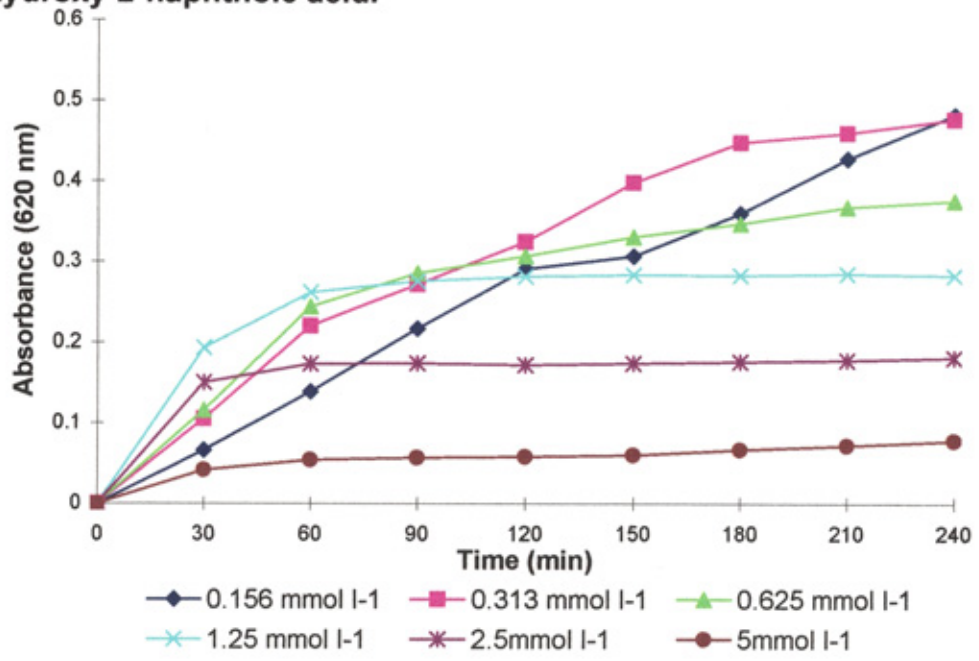


Figure 2.10.3: Generation of indamine by a range of organisms due to hydrolysis of L-alanyl-DEPPD (2.5 mmol l^{-1}) in the presence of 3,5-dihydroxy-2-naphthoic acid at 0.625 mmol l^{-1}.

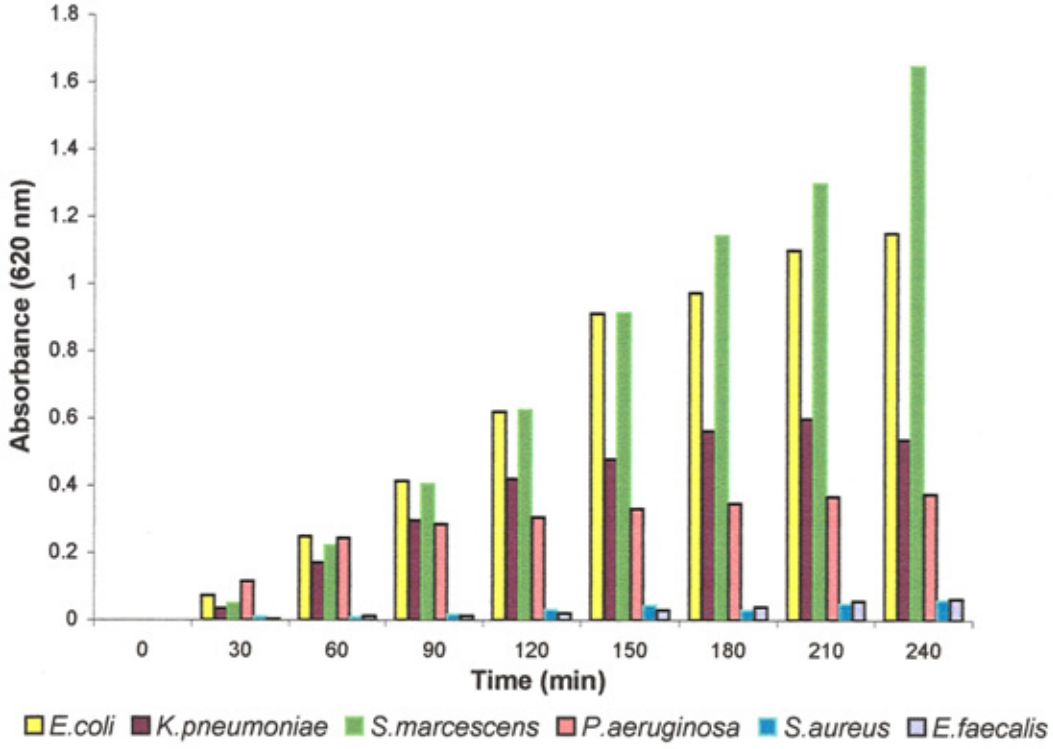
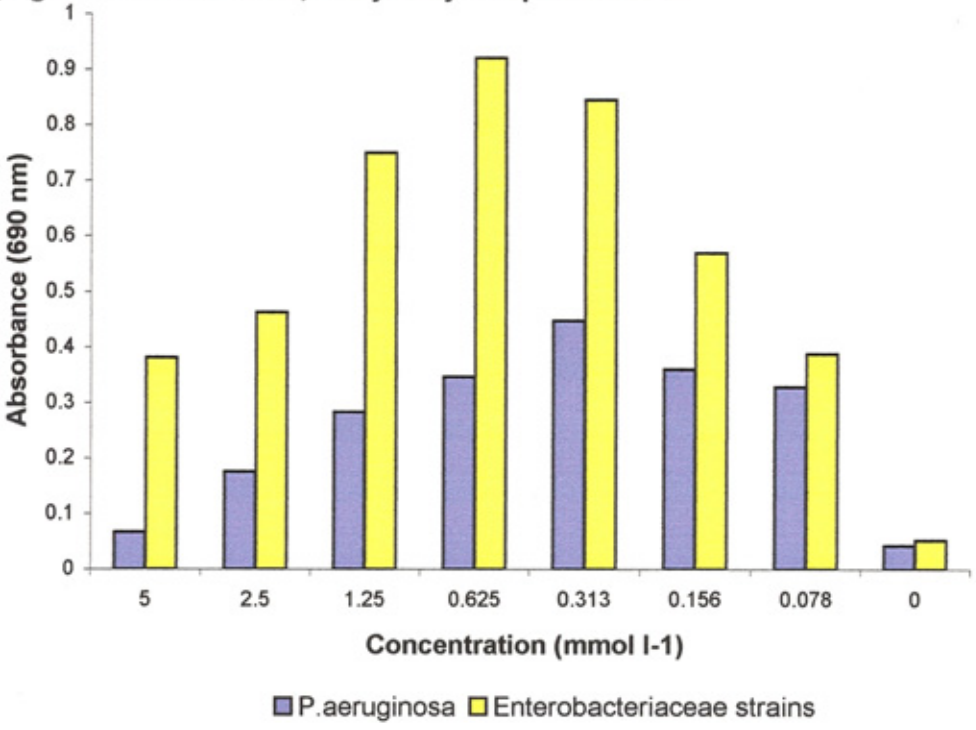


Figure 2.10.4: Average increases in absorbance produced by Enterobacteriaceae strains and *P.aeruginosa* over three hours due to hydrolysis of L-alanyl-DEPPD (2.5 mmol l^{-1}) in the presence of varying concentrations of 3,5-dihydroxy-2-naphthoic acid.



Determination of the colour produced by *E.coli* in the presence of five L-alanyl and six naphthol derivatives.

The results of this experiment are shown in Figure 2.11. Both *p*-phenylenediamine substrates reacted well with several naphthols. The most intense coloured complexes developed with 1-naphthol (A:1 and B:1), 3,5-dihydroxy-2-naphthoic acid (A:3 and B:3), 4-chloro-1-naphthol (A:7 and B:7), and 5[[(1-hydroxy-2-naphthyl)-carbonyl]octadecylamino]-isophthalic acid (A5: and B:5). Of the two substrates the stronger reaction occurred with the diethyl derivative for all of the naphthols tested.

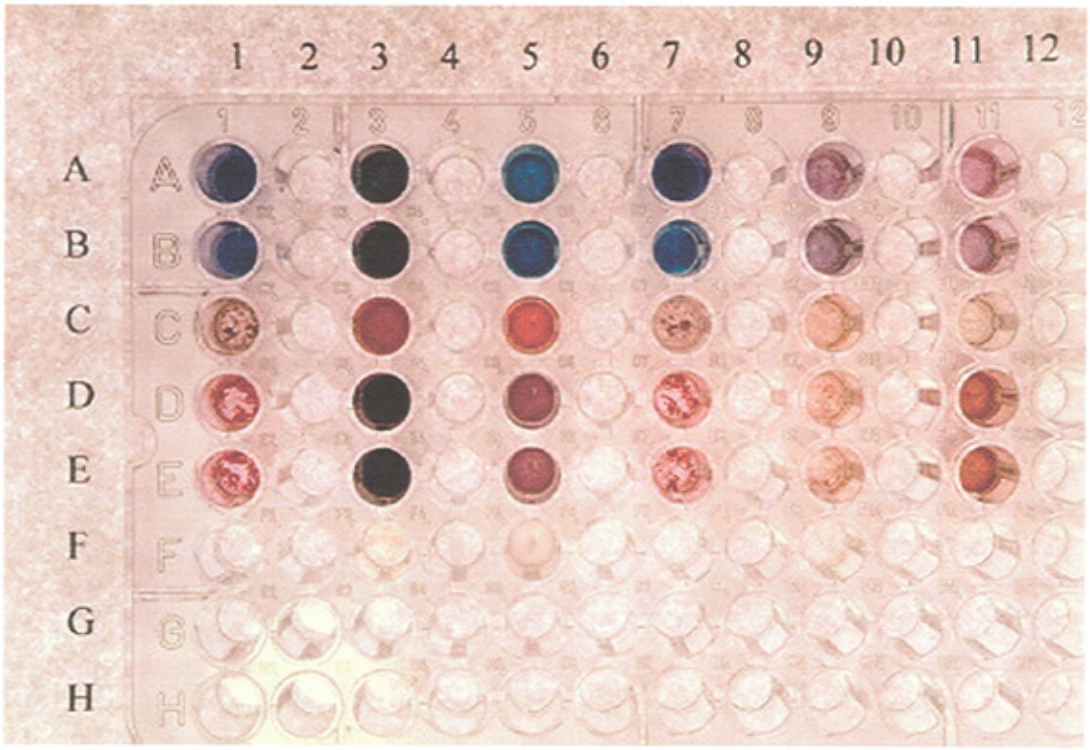


Figure 2.11. Colour development of five naphthols (0.625 mmol l^{-1}) in the presence of six L-alanyl derivatives (2.5 mmol l^{-1}) and *E.coli* (NCTC 10418).

Row A	L-alanyl-DMPPD
Row B	L-alanyl-DEPPD
Row C	L-ala-4-aminophenol
Row D	L-alanyl-4-amino-2,6-dibromophenol
Row E	L-alanyl-4-amino-2,6-dichlorophenol

(Row F is substrate free control i.e. naphthol plus organism and buffer)

Column 1	1-naphthol
Column 3	3,5-dihydroxy-2-naphthoic acid
Column 5	5[[(1-hydroxy-2-naphthyl)-carbonyl]octadecylamino]-isophthalic acid.
Column 7	4-chloro-1-naphthol
Column 9	anthranol
Column 11	Substrate plus organism plus buffer only.

(Even numbered columns contain buffer plus naphthol only).

For the 4-aminophenol derivatives tested, L-alanyl-4-aminophenol produced a intense reaction in the presence of 3,5-dihydroxy-2-naphthoic acid (C:3), and very weak results with 1-naphthol (C:1) and 4-chloro-1-naphthol (C:7). Both halogenated derivatives produced strong reactions in the presence of 3,5-dihydroxy-2-naphthoic acid (D:3 and E:3), 1-naphthol (D:1 and E:1), and 4-chloro-1-naphthol (D:7 and E:7), with the latter two forming a red/purple precipitate in the well. Anthranol reacted slightly better in the presence of the di-chloro derivative (E:9). However, the wells containing organisms plus substrate and buffer (A: 11 - E: 11) produced coloured reaction products. For the *p*-phenylenediamines these were a red/purple colour. Strong orange coloured products were observed in the halogenated aminophenol wells. These reactions were not as intense as those observed with 3,5-dihydroxy-2-naphthoic acid. These coloured products in the presence of buffer alone are presumably the oxidised compounds formed on exposure to air.

Overall these results have largely mirrored those of the previous experiment (Fig 2.3) where core compounds rather than substrates were examined. The most intensely coloured substrate/naphthol complexes occurred with both *p*-phenylenediamines, although the diethyl derivative was better, due to its enhanced solubility. Using these derivatives the optimal choice of naphthol would be either 1-naphthol or 3,5-dihydroxy-2-naphthoic acid, both producing intensely coloured reaction products. For 4-aminophenols the

halogenated derivatives both produced equally intense reactions with 1-naphthol and 3,5-dihydroxy-2-naphthoic acid. Again due to the superior solubility of the dichloro derivative this compound should prove useful in the production of a wide range of substrates. Additionally L-alanyl-4-aminophenol can be considered an important substrate, but only when coupled with 3,5-dihydroxy-2-naphthoic acid.

Determination of the optimal concentration of 3,5-dihydroxy-2-naphthoic acid for the production of colour using 4-aminophenyl-β-D-glucuronide.

The results of this experiment are shown in Figure 2.12. Strong reactions occurred in the range of wells containing 2.5 mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid (Row D) and substrate concentrations of between 2.5 - 0.625 mmol l^{-1} (Columns 2-4). As judged visibly the most intense colour was observed in the well containing 2.5 mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid, and 2.5 mmol l^{-1} 4-aminophenyl-β-D-glucuronide (D:2). Thus, these were the optimal concentrations of these two compounds for the development of coloured products. These concentrations were used in subsequent experiments (Chapter 3) in liquid media for testing all substrates for the detection of glycosidases and phosphatases.

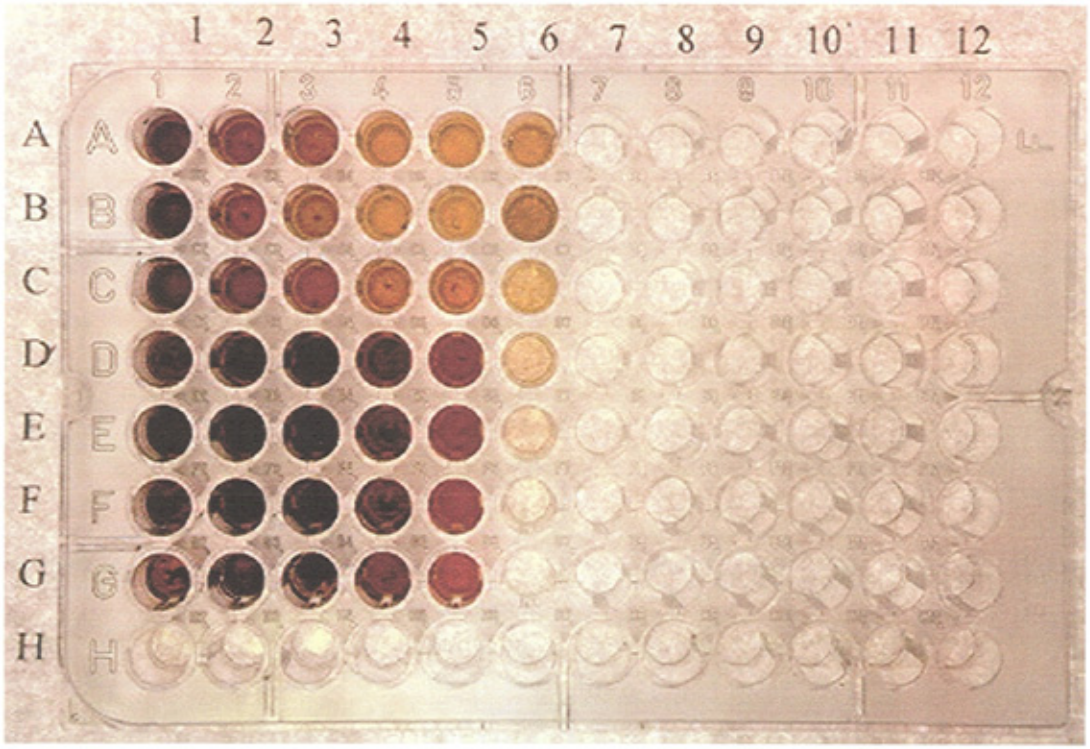


Figure 2.12. Showing a "chequerboard" titration. Rows A - G contain concentrations of 3,5-dihydroxy-2-naphthoic acid from 20 – 0.325 mmol l^{-1} in a two-fold doubling dilution series. Columns 1-5 contain concentrations of 4-aminophenyl-β-D-glucuronide from 5 – 0.325 mmol l^{-1} in a doubling dilution series using *E.coli* (NCTC 10418). Row H contains substrate plus buffer only, and column 6 contains naphthol plus buffer only.

Production of absorption spectra for the various complexes formed between aminophenol, *p*-phenylenediamines and naphthols.

A summary of the absorbance maxima for each coloured complex is shown in Table 2.2. For DEPPD, in coupling with both 1-naphthol and 3,5-dihydroxy-2-naphthoic acid, the absorbance was identical, at 575 nm.

This absorbance would be expected to produce a blue-violet colour (Finar, 1973), and indeed this was the colour observed in previous experiments (Fig 2.3). DMMPD had a slightly higher absorbance maximum (590 and 580nm). In practice, however, the coloured complex observed with both DEPPD and DMPPD could not be differentiated visibly. The compound 4-amino-2,6-dichlorophenol and both naphthols produced a similar absorption maxima (560 and 575 nm) to those observed with *p*-phenylenediamines. Indeed the colour observed visibly was identical for that observed with both DEPPD and DMPPD. Coupling between both naphthols and 4-aminophenol produced an orange complex as observed in previous experiments. The absorbance maxima for both was 475 nm, and this would equate with a yellow-orange colour. Coupling between 4-aminophenol and 4-chloro-1-naphthol gave rise to a compound, which absorbed maximally at around 475 nm. The expected colour of this compound would be yellow orange and this was observed in Figure 2.3.

Table 2.2 Approximate absorbance maxima (nm) for each Indamine/Indophenol complex.

Indamine/Indophenol complex	Absorbance maxima (nm)
4-amino-2,6-dichlorophenol/1-naphthol	560
4-amino-2,6-dichlorophenol/3,5-dihydroxy-2-naphthoic acid	575
4-aminophenol/1-naphthol	475
4-aminophenol/3,5-dihydroxy-2-naphthoic acid	475
DEPPD/1-naphthol	575
DEPPD/3,5-dihydroxy-2-naphthoic acid	575
DMPPD/1-naphthol	590
DMPPD/3,5-dihydroxy-2-naphthoic acid	580

Evaluation of a range of naphtholic derivatives as alternatives to naphthol for the formation of coloured complexes with DEPPD.

Figure 2.13 shows the coloured reaction products formed in this experiment.

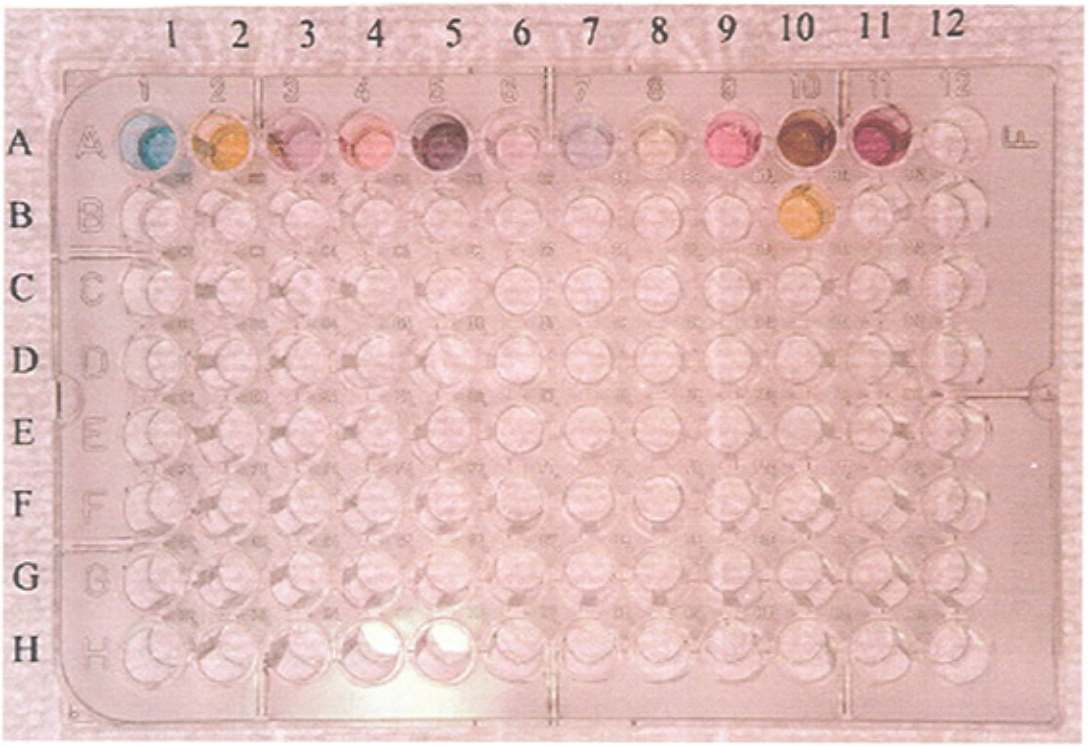


Figure 2.13. Colour produced by 10 naphthol derivatives (5 mmol l^{-1}) in the presence of 5 mmol l^{-1} DEPPD. Row A (wells 1-11) contained the following: 1: phloroglucinol anhydrous, 2: naphthol AS, 3: phenyl-5-isoxazolone, 4: 3-methyl-2-benzothiazolone hydrazone hydrochloride, 5:resorcinol, 6: 3-methyl-1-phenyl-2-pyrazoline-5-one, 7: barbituric acid, 8: 1-hydroxyisoquinoline, 9: rhodanine-3-acetic acid, 10: di-isoproterenol dihydrochloride. Well 11 contained buffer and DEPPD only. Row B contained naphthol and buffer only.

No colour was produced in control wells except for di-isoproterenol dihydrochloride which was yellow (B:10). This derivative produced a brown colour upon reaction with DEPPD (A:10). All compounds tested produced a coloured complex in reaction with DEPPD. As can be observed most of the coloured complexes are very weakly coloured, suggesting that none of these compounds offer any advantages over 3,5-dihydroxy-2-naphthoic acid for coupling with *p*-phenylenediamines or aminophenols, since reaction of these core compounds and this napthol have been shown to produce intensely coloured reaction products (Fig 2.3*)*.

Discussion

The preliminary experiments detailed in this Chapter, clearly demonstrate that coupling occurred between *p*-phenylenediamines, and 1-napthol thus validating the "Nadi reaction". Additionally, coupling also occurred between the structurally similar aminophenols and a variety of other naphthols. One of the most interesting features was the development of a red colour in the control wells containing each *p*-phenylenediamine derivative (Fig 2.2). These are the oxidised forms known as Wursters salts, for which no structural formula has yet been proposed (Michaelis & Granick, 1943). It has been suggested that this auto-oxidation is due to the presence of trace amounts of heavy metals, or more likely, catalysed by the presence of organic impurities, not removed during preparation, reacting with dissolved oxygen (Nachals *et al.*, 1958). Since the Nadi reaction occurs spontaneously in the presence of atmospheric oxygen its validity as a histochemical indicator has been questioned. This has led to the investigation of other more stable-coupling agents e.g. 4-amino-1-N-N-dimethylnaphthylamine (Nachals *et al.*, 1958). Since cytochrome oxidase is not required for the coupling of naphthol and *p*-phenylendiamine, released on substrate hydrolysis, the system could be used to detect enzyme hydrolysis of a derivative of one of these substrates, in organisms, which lack cytochrome oxidase activity.

It was found that the colour and intensity of the reaction products depend upon the ability of the naphthol to couple and form a stable coloured complex. The colour was best demonstrated at or around neutral pH, a finding in agreement with Clark (1972). It was interesting to note that Clark found in acid conditions (pH 4-5) that indophenols tended to precipitate, and at alkaline pH, decomposition occurred. In addition it was found that substitution of halogen atoms into the indophenol nucleus reduced pK_0 so that these derivatives retained a strong blue colour even in acidic conditions. It was evident from the experiments of the present study that the halogenated compounds of 4-aminophenol e.g. 4-amino-2,6-dichlorophenol (Fig 2.14) produced intensely coloured reaction products.

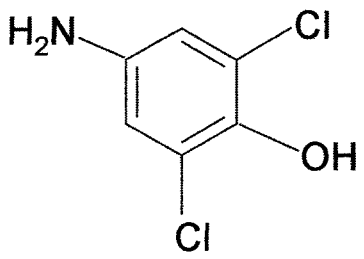
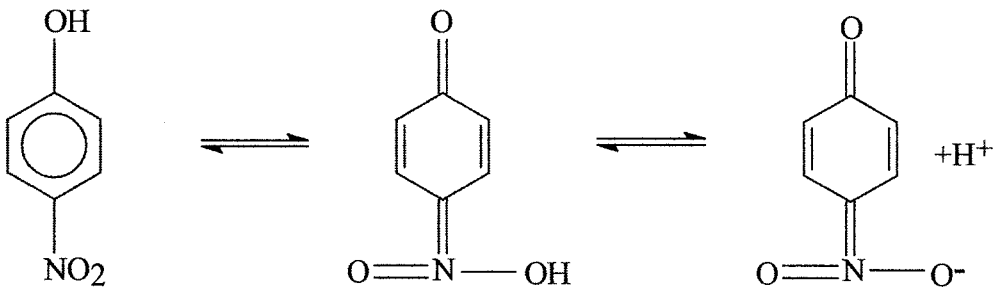


Figure 2.14. Structure of 4-amino-2,6-dichlorophenol.

The fact that the colour development is optimal at neutral pH lends an advantage over chromogens such as *o*- and *p*-nitrophenols (Fig 2.15), where the yellow colour development is optimal at an alkaline pH (Bascomb, 1987).



Colourless form in acid medium

yellow acid-forms which dissociates in alkaline medium.

Figure 2.15. Diagram of the structural changes of *p*-nitrophenol with pH.

The pH transition level of this compound lie between 5.6 and 7.8, the colour intensity reaches a maximum at pH 7.6, which can be detected visually or by measuring absorbance at 405 nm.

It is unusual for culture media, particularly solid media, to be at the optimal pH for the detection of a released chromogen such as *p*-nitrophenol. It routine practice most culture media are maintained at or around neutral pH for the optimal isolation of bacterial pathogens.

The reactions investigated in this project differ markedly from the detection of bacterial enzyme hydrolysis using conventional chromogenic substrates, where substrate hydrolysis usually yields one or more reaction products which are visible and do not require the addition of reagents for the development of a strongly coloured product. However, there are exceptions,

for example, the detection of substrates based on *p*-nitroanilide (*p*-NA), since the released chromogen is poorly coloured (Dealler, 1993). The reaction product of *p*-NA based substrates can be enhanced by using *p*-dimethylaminocinamaldehyde (DCMA). Care must be taken, however, as the reagent is prepared in hydrochloric acid at pH 1.0. This reagent is also used to develop the reaction of substrates based on β-naphthylamine (James, 1994). In addition, *p*-NA and *o*-nitrophenol *(o*-NP) substrates cannot be used in agar-based media due to the diffusion of the released chromogen throughout the medium (Manafi, 1996), and to the toxicity that would occur if the medium were at pH 8.0. The fact that the novel compounds examined in the present study show optimal colour development at pH 7.2, and are of low toxicity offers a distinct advantage over *p*-NA and *o*-NP. Moreover the intensity of the colour produced by coupling of *p*-phenylenediamines and aminophenols is more intense than that generated by either *p*-NA or *o*-NP.

Very little data has been produced regarding the toxicity of chromogenic and fluorogenic compounds, presumably since they are most often used for studies in liquid media where the test objectives are to produce rapid results, using heavy inocula. Thus any toxicity effects are likely to be minimised due to the number of micro-organisms present, typically $>10^9$ per test. Kayser & Kolodziej (1999) showed the antibacterial effects of a range of simple coumarins (Fig 2.16) showing that the degree of antibacterial activity correlated with the number of oxygen substituents of the coumarin ring.

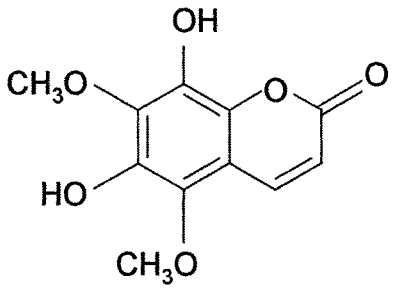


Figure 2.16. Chemical structure of 6,8-dihydroxy-5-7-dimethoxycoumarin.

The presence of a hydroxyl group at the 7-position of the coumarin ring significantly reduced the antibacterial activity of the coumarin. A methoxy substitution at position 7 increased the antibacterial activity. The effect of different substitutions is highly relevant to the toxicity of both core compounds and naphthols. It has been suggested that the avoidance of bulky side chain substitution and simple aromatic substitution, aid in penetration of bacterial cell walls (Rauckman *et al.*, 1989).

In the experiments of the present study, the lack of toxicity of aminophenol and derivatives was surprising, since phenol and especially the chlorinated forms are simple aromatics and would be expected to penetrate bacterial cells easily. Many phenolic compounds are potent bactericides (McDonnell and Russell, 1999). For example, phenol at relatively low concentrations e.g. 320 mg l^{-1} rapidly lyses growing cultures of *E.coli*, Staphylococci and

Streptococci (Pulvertaft and Lumb, 1948) inducing a progressive leakage of intracellular components (Lambert and Hammond, 1973). Chlorinated phenolics e.g. 2,4,6-trichlorophenol (Fig 2.17) are also potent bactericides and are similar in their membrane damaging effects (Hugo, 1971).

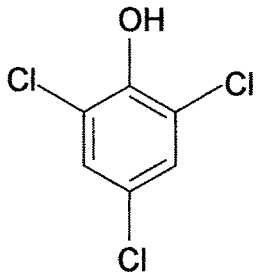


Figure 2.17. Chemical structure of 2,4,6-trichlorophenol

The highest concentration of any core compound tested was 0.313 mmol l^{-1} which was equivalent to 34 mg l^{-1} for 4-aminophenol to 84 mg l^{-1} for the highest molecular weight di-bromo derivative. 4-aminophenol was inhibitory at much lower concentrations than those observed for phenol at an equivalent concentration by Pulvertaft and Lumb (1948) for *E.coli*, yet the di-bromo compound was relatively non-inhibitory. The reason for the lack of toxicity of the latter compound has yet to be determined.

The toxicity of naphthols was as expected, since naphthol and the 4-chloro derivative were highly toxic. Again the simple aromatic structure would facilitate cell penetration (Rauckman *et al.*, 1989) and promote intracellular

damage. Similarly the lack of toxicity of 3,5-dihydroxy-2-naphthoic acid may in part by be explained by the presence of both a carboxyl acid group and a hydroxyl group, which have been demonstrated to significantly reduce antimicrobial activity when compared to the parent compound (Kayser and Kolodziej, 1999). The compound 5[[(1-hydroxy-2-naphthyl)-carbonyl]octadecylamino]-isophthalic acid with its extended side chains was relatively non-toxic towards the Gram-negative strains tested (Table 2.1). The bulky side chain structure of this compound would have difficulty in penetrating bacterial cell walls and this could account for its lack of toxicity compared to the simple aromatic naphthols (Kayser and Kolodziej, 1999).

The toxicity of L-alanyl substrates was similar to that of the core compounds. *S.aureus* was inhibited significantly by three of the L-alanyl substrates tested. The exceptions were L-alanyl-4-aminophenol and L-alanyl-DMPPD, which were relatively non-toxic.

The coupling of an amino acid with a core compound would be expected to have some effect on cell toxicity, although since Gram-positive organisms would fail to hydrolyse the substrate, the toxicity towards *S.aureus* must be entirely related to the substrate itself rather than to any product. Alternately it is possible that impurities generated in substrate synthesis may account for some toxic effects. It would be envisaged that toxicity towards Gram-negative cells would reflect that observed with core compounds (products of

enzyme hydrolysis) since production of the relevant aminopeptidase would release the core compound, and indeed this was observed (Figures 2.7.1-2.7.11)

The development of a useful system for the identification of clinically significant microorganisms requires a system whereby the substrates show high solubility in water, are easily hydrolysed by target enzymes and produce intensely coloured reaction products upon coupling that can be used in both liquid and solid media due to their low toxicity. Similarly the coupling naphtholic compound must also be highly soluble and non-toxic to a wide range of target organisms. Therefore from the results of these experiments the optimal *p*-phenylenediamine derivative would be the diethyl form, which showed improved solubility over the di-methyl-compound. Of the 4-aminophenols the di-chloro derivative showed good solubility, and the intensity of the coupling product would suggest this to be the most useful aminophenol for further studies. Moreover the most appropriate naptholic compound would appear to be 3,5-dihydroxy-2-naphthoic acid which produced intensely coloured reaction products, was relatively non-toxic, and had enhanced solubility compared to 1-naphthol. The lack of toxicity of these compounds (DEPPD and 3,5-dihydroxy-2-naphthoic acid) is of increased significance in relation to studies performed in agar-based media, as discussed in Chapter 4.

These experiments have shown that 4-aminophenol and *p*-phenylenediamine compounds coupled with 1-naphthol and its derivatives produce intensely coloured products of relatively low toxicity, with the reaction easily visible in 4 hours. In these respects, these compounds meet all of the requirements of a universal reaction system for use in both solid and liquid media. Derivatives of the most promising of these compounds, will be examined further using 3,5-dihydroxy-2-naphthoic acid as a coupling agent, in Chapters 3 and 4.

Chapter 3

Examination of substrates based on *p*-phenylenediamines and 4-aminophenol for the detection of bacterial hydrolyases in liquid media.

Introduction

Most of the early work in the identification of microorganisms was confined to the use of pH indicators to detect products of fermentation (Cowan, 1968). However, this often requires overnight incubation, before the acidic end products can be detected visibly (Chen *et al.*, 2000). Carbohydrate fermentation tests are still the most widely used methods for the identification of microorganisms, mainly by use of commercial kit systems (De Paulis *et al.*, 2003). Most early pH indicators, e.g. phenol red are adequate for detection of pH change in liquid media (Sado *et al.*, 1988), and are still employed despite the development of numerous more sensitive pH indicators designed to speed up the detection of bacterial fermentation e.g. Nitrazine yellow.

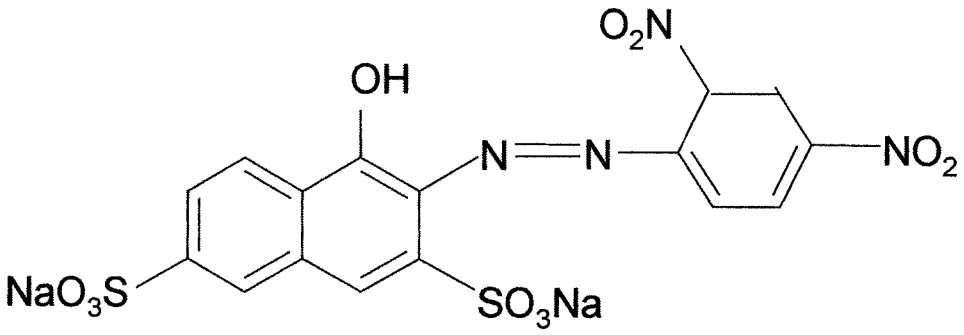


Figure 3.1. Structure of 2-(2,4-dinitrophenylazo)-1-hydroxynapthylene-3,5-disulphonic acid, disodium salt (Nitrazine yellow). This indicator produces a sharp colour change from yellow to blue over the narrow pH range of 6.0-7.2.

This typifies many of the procedures used for bacterial identification, in that despite numerous novel developments, few are implemented in diagnostic microbiology (James, 2000).

Of even greater sensitivity are fluorescent pH indicators, which are usually based on fluorescein or 7-hydroxy-4-methylcoumarin (Haughland, 1996). The change in pH is determined by a change in the excitation and emission frequency of the fluorogen during pH changes. Fluorescein, is a broad pH range indicator (typically pH 4-9) but it has the disadvantage of having a small Stokes shift and is therefore prone to interference from stray light, limiting its use (Wolfbeis and Marhold, 1987). Moreover, fluorescein and other fluorescent indicators suffer from photobleaching (Haugland, 1996). Combined with the requirement of a UV light source for detection, these problems have limited the use of fluorescent pH indicators, despite a high degree of sensitivity.

More recently bacterial identification tests have involved a shift away from detection by pH change, and the complicated metabolic pathways involved, towards the detection of individual enzymes. In particular substrates for glycosidases and aminopeptidases, using chromogenic labels in particular (Grenier *et al.*, 2001), continue to be developed.

Perhaps, the most widespread use of substrates has been for the detection of glycosidase enzymes. These are involved in the breakdown of exogenous natural products to supply carbon and energy to a cell (Beguin *et al.*, 1992). In addition, such enzymes have a role in the synthesis of cell-wall polysaccharides and nucleotide synthesis (Bascomb, 1987). The best characterised of the oligosacharide-hydrolysing enzymes is the inducible β-galactosidase of coliform bacteria. Numerous substrates are available for the detection of glycosidases, of which the most widely used are based on 4-methylumbelliferone (4-MeU), *p*-NP, *o*-NP, or fluorescein (Figure 3.2), all of which are commercially available (Homer *et al.*, 2001).

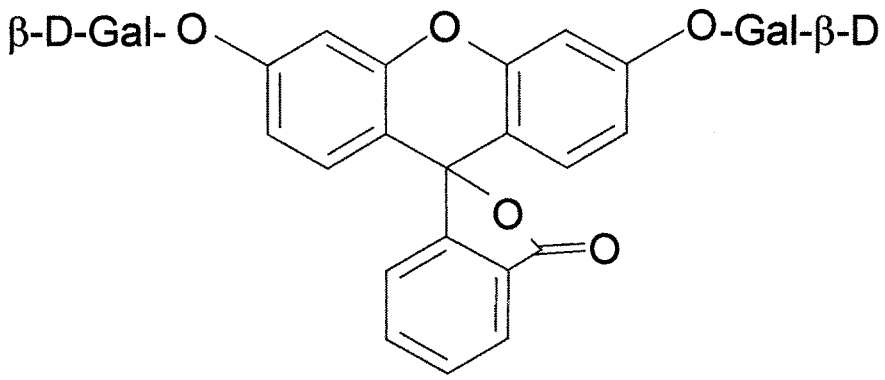


Figure 3.2. Structure of Fluorescein di-β-D-galactopyranoside. The substrate is first hydrolysed to the mono form and then to the highly fluorescent compound fluorescein by β-galactosidase (Haughland, 1996).

One of the most widely used synthetic chromogenic substrates is the colourless *o*-nitrophenyl-β-D-galactopyranoside (ONPG) which when hydrolysed by β-galactosidase releases the pale yellow *o*-NP (Apartin and Ronco, 2001). Wallenfels and Malhotra (1961) studied the substrate specificity of the enzyme β-galactoside from *E.coli* using synthetic derivatised nitrophenol (NP) substrates and the natural substrate lactose. The authors demonstrated a 20-30 fold higher activity of the enzyme, β-galactoside, towards ortho (*o-)* and para (*p-)* substituted nitrophenol-β-D-galactopyranoside, than towards lactose. Le Minor and Ben Hamida (1962) first recommended the use of *p*-nitrophenol-β-D-galactoside for the differentiation of late lactose-fermenting species e.g. *E.coli* A-D group, from lactose negative organisms such as *Salmonella* spp. Other substrates include both α and β isomers of monosacharides such as glucose, fucose, and xylose; and the disaccharides lactose and maltose. These compounds have been applied for the detection of glycosidase activity in several bacterial genera, including enterobacteriaceae. Marin and Marshall (1983) studied the glycosidase activity of psychrotrophic bacteria using eleven *p*-NP substrates, and later Killian and Bulow (1976) performed a similar study for enteric bacteria.

The strong electron-withdrawing properties of the nitro group enhances enzyme hydrolysis rates of nitrophenol based substrates (Haugland, 1996),

which may confer an advantage over the substrates tested in this study. Such substrates offer numerous advantages over the "natural" substrate, being both highly reactive and sufficiently sensitive for the rapid detection of a range of enzyme activity in microorganisms (James, 1994).

The other major groups of enzymes involved in bacterial identification are the aminopeptidases (Takahashi *et al.*, 2002). These form a group known as exopeptidases, which catalyse the stepwise cleavage of an amino acid from the amino terminal of a peptide (Asano, 1989). These enzymes, which are present in mammalian, bacterial and fungal cells, have specificities that are radically different, suggesting an evolutionary divergence (Gasparello-Clamente and Silveira, 2002). They can therefore be used to distinguish closely related species and even strains of the same species (Watson, 1976). The most widely used substrates for the detection of aminopeptidase activity are those of 7-AMC; Figure 3.3, although fluorogenic leaving groups have also been used e.g. 6-aminoquinoline (Brynes *et al.*, 1981, Yoshikawa *et al.*, 1996). Godsey *et al.*, (1981) first described the use of 7-AMC substrates for the detection of bacterial aminopeptidase activity and these are now widely available for detection of various bacterial peptidases (Holliday *et al.*, 1999).

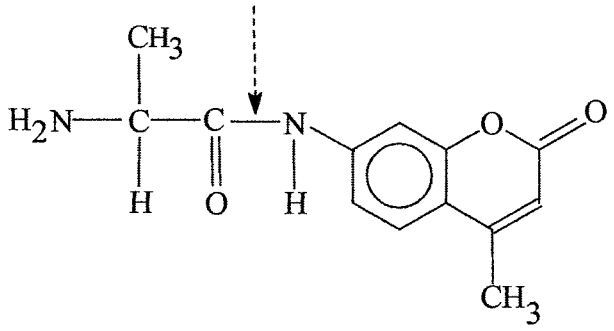


Figure 3.3. Structural formula for L-alanyl-7-amido-4-methylcoumarin showing the site of enzyme hydrolysis (dotted arrow). Upon enzyme hydrolysis, blue fluorescence of 7-AMC is observed under UV light.

For the chromogenic detection of enzymes hydrolysing peptide bonds, the most common substrates are based on β-NAP or *p*-NA (Cahan *et al.*, 2001) the latter releasing the yellow *p*-nitroaniline upon hydrolysis (Fig 3.4). Similarly to *p*-NP substrates, results can be recorded visually or by measurement of absorbance. Since β-NAP substrates release the carcinogenic β-naphthylamine upon hydrolysis (Connolly and White, 1969), most applications use either *p*-NA or *p*-NP substrates if a chromogenic label is preferred.

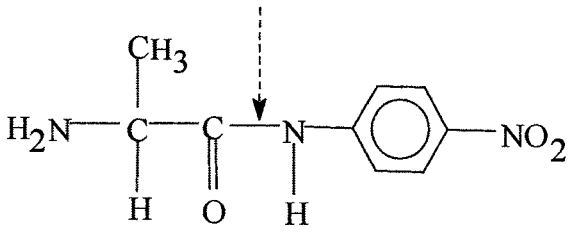


Figure 3.4. Structural formula for L-alanyl-*p*-nitroanilide showing the site of enzyme hydrolysis (dotted arrow), to release *p*-nitroaniline.

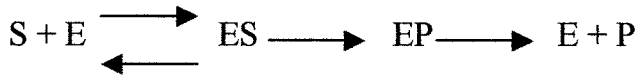
The ability to interpret the reaction products visually is important, as the requirement for expensive instrumentation is overcome, making the diagnostic use of chromogenic substrates more attractive. Studies have shown many chromogenic substrates to be more sensitive than their fluorescent counterparts in solid media, although in liquid studies sensitivity rarely approaches that achievable using 4-MeU or 7-AMC (Doleans, 1994).

Numerous other enzymes are exploited for the purposes of routine bacterial and fungal identification e.g. phosphatases (Eisgruber *et al.*, 2003), esterases (Adcock and Saint, 2001), and lipases (Buzzini and Martini, 2002). A wide range of substrates are available commercially, the chromogenic or fluorogenic labels are the same as those used for glycosidase detection (Bascomb, 1987).

Enzymes are globular proteins and are extremely efficient at accelerating chemical reactions towards equilibrium (Stryer, 1995). The functional

characteristics of these proteins is that they can bind one or more substrate molecules, the binding of which, takes place in a hydrophobic cleft known as the active site (Apps *et al.*, 1992). An enzyme-catalysed reaction proceeds between 10^3 to 10^{17} times faster than an uncatalysed reaction (Murray *et al.*, 1993).

The model for explaining the kinetics of an enzyme-catalysed reaction was proposed by Michaelis and Menten (Stryer, 1995). They proposed that an enzyme transiently binds to a substrate to form an enzyme-substrate complex [ES], the rate of which depends upon the concentrations of both substrate and enzyme. At the beginning of an enzyme-catalysed reaction, the amount of product formed can be described by the equation:-



Equation 3.1 Rate constant of a reaction with a single substrate (S), and single product (P), enzyme (E) reaction. (Reed *et al.*, 1998).

During the initial reaction little product is formed, thus the rate of reaction is small. The velocity of the reaction during this stage is called the initial velocity (V_o). Use of this simplifies the interpretation of any data derived from kinetic experiments that may arise as the reaction progresses, such as

depletion of substrate, possible slow enzyme denaturation or product inhibition (Murray *et al.*, 1993). For enzyme reactions, which obey Michaelis-Menten kinetics, initially the determination of both K_m and V_{max} are important. V_{max} is attained when the enzyme is saturated with substrate, and K_m is a measure of the affinity of an enzyme for its substrate, expressed in terms of substrate concentration (Apps *et al.*, 1992). It follows that an enzyme with a high K_m requires a higher substrate concentration to achieve a given velocity than an enzyme that has a low K_m. Fumarase which catalyses the reaction of fumarate to malate has a K_m of 5×10^{-6}mol l^{-1} indicating effective catalysis can occur even at extremely low substrate concentrations (Anon, 2000). Both K_m and V_{max} are derived from the Michaelis-Menten equation: -

$$V = \frac{V_{max}\ [S]}{K_m + [S]}$$

Equation 3.2 The Michaelis-Menten equation.

The Michaelis constant K_m and the maximal enzyme rate V_{max} can be derived from experiments determining the rates of catalysis at different substrate concentrations, provided the enzyme functions according to the simplified scheme of Michaelis and Menten (Murray *et al.*, 1993). Far more conveniently, input of experimental data into the Michaelis-Menten

equation can be transformed to produce a straight line plot, by taking the reciprocal of both sides of the equation. A plot of 1/V versus 1/[S] produces the Lineweaver-Burk plot (Equation 3.3), yielding a straight line with the intercept of $1/V_{max}$ and a slope of K_m/V_{max}. Normally this is generated using commercially available enzyme software packages e.g. Enzpack (Biosoft Ltd).

$$V = \frac{V_{max}\ [S]}{K_m + [S]} \qquad \frac{1}{V} = \frac{K_m}{V_{max}\ [S]} \cdot \frac{1}{V_{max}}$$

Equation 3.3 Michaelis-Menten equation transformed to give Lineweaver-Burk plot.

The enzyme kinetic experiments in this thesis require the comparison of rates of substrate hydrolysis using identical concentrations of purified enzyme. By keeping the reaction conditions for the novel substrates identical, a direct comparison with ONPG can be made using the enzyme β-galactosidase, derived initially from K_m. The best method of comparison of substrate hydrolysis rates would be to use the constant K_{cat}, which is a direct measure of the catalytic production of product under optimal conditions (Apps *et al.*, 1992), the reciprocal of which can be considered as the time required by an enzyme to "turn over" one substrate molecule, or alternately K_{cat} measures the number of substrate molecules turned over per

enzyme molecule per second. By deriving this value initially from the Michaelis-Menten equation e.g. $V_{max} = K_{cat}\,[E]_T$ where $[E]_T$ is the concentration of the enzyme the value for K_{cat} can be derived. Furthermore, a plot of K_{cat}/K_m reveals the efficiency of the enzyme for a particular test substrate. These values have been determined for ONPG, 4-aminophenol-β-D-galactoside and 4-amino-2,6-dichlorophenyl-β-D-galactoside and as such provide a comparison of how useful these substrates are for the detection of bacterial hydrolyases.

The enzyme β-galactosidase is widely distributed throughout nature, and numerous publications are available on its structures and functions (Jedrzejas, 2000). Since purified β-galactosidase is an important diagnostic enzyme, and is readily available, it was logical to examine novel galactoside substrates in comparison with the most commonly used chromogenic substrate ONPG. The comparison of new chromogenic substrates to determine K_m and V_{max} was used by Pócsi *et al.*, (1992) for 5 novel substrates using different β-galactosidase enzymes. These substrates were based on 4-[2-(4-β-D-galactopyranosyloxy-3-methoxyphenyl)-vinyl], the methylquinolinium iodide derivative is shown in Figure 3.5.

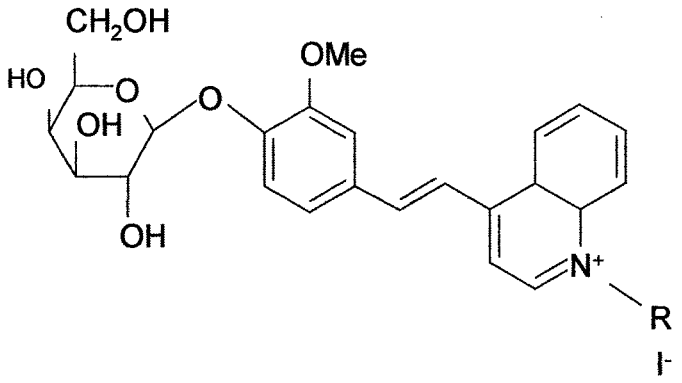


Figure 3.5. Chemical structure of 4-[2-(4-β-D-galactopyranosyloxy-3-methoxyphenyl)-vinyl]-1-methylquinolinium iodide.

It was found that with *E.coli* β-galactosidase, all substrates attached tightly to the active centre of the enzyme, a situation that resulted in low K_m values. The authors also suggested that the differing heterocyclic portions of the aglycones had no effect on substrate specificity (Pócsi *et al.*, 1992). Whilst the rates of substrate hydrolysis are important, it must be remembered that the entry of substrate into the cell and the subsequent release of the chromogenic moiety are also important in the production of a coloured reaction product.

For the purposes of this thesis the kinetic parameters for β-galactosidase using novel 4-aminophenol substrates have been determined using purified *E.coli* β-galactosidase and compared with those of ONPG. The main part of this Chapter has been the examination of several novel substrates in

comparison with commercially available chromogenic alternatives in liquid format.

Materials

Cultures

Bacterial and fungal strains from NCTC, ACTC, or NCPF, purchased from Central Public Health Laboratory, Colindale, London, were used in experiments for the detection of enzymes in liquid media. All organisms were freshly subcultured for use in all experiments on Columbia agar base supplemented with 5% v/v horse blood and checked for purity before experimental use. A stock collection of selected bacteria was used in all experiments except those for the detection of staphylocoagulase, where several wild strains of *S.aureus* isolated from blood cultures were used. In addition these strains were used for the production of photographs of experimental results where appropriate. These were:- *Escherichia coli* NCTC 10418, *Klebsiella pneumoniae* NCTC 10896, *Providencia rettgeri* NCTC 7475, *Enterobacter cloacae* NCTC 11936, *Serratia marcescens* NCTC 10211, *Salmonella typhimurium* NCTC 74, *Pseudomonas aeruginosa* NCTC 10662, *Staphylococcus epidermidis* NCTC 11047, *Streptococcus pyogenes* NCTC 8306, *Enterococcus faecalis* NCTC 755, *Enterococcus faecium* NCTC 7171, *Listeria monocytogenes* NCTC 11994, *Staphylococcus aureus* NCTC 6571, *Proteus mirabilis* NCTC 10975, and *Yersinia enterocolitica* NCTC 11176.

In addition a range of other control strains were used depending upon the test substrate. These were:- *Bacillus cereus* NCTC 8143, *Haemophilus influenzae* NCTC 8143, *H.influenzae* NCTC 11931, *H.influenzae* NCTC 8467, *H.influenzae* NCTC 11315, *Haemophilus parainfluenzae* NCTC 7857, *Aeromonas caviae* NCTC 10852, *Aeromonas hydrophila* NCTC 8049, *A.hydrophila* NCTC 3249, *Aeromonas sobria* NCTC 11215, *Candida albicans* ATCC 90028, *C.albicans* ATCC 90029, *C.albicans* ATCC 64547, *C.albicans* ATCC 64551, *C.albicans* NCPF 3281, *Candida glabrata* NCPF 3943, *C.glabrata* NCPF 8018, *Candida krusei* NCPF 3945, *Candida lusitaniae* NCPF 3945, and *Candida parapsilosis* NCPF 3980, *Pseudomonas stutzeri* ATCC 17588T, *Burkholderia cepacia* ATCC 27511T, *Brevundimonas vesicularis* ATCC 114426T, *Pseudomonas fluorescens* ATCC 13525, *Pseudomonas alcaligenes* ATCC 14909T, *Shewanella putrefaciens* ATCC 8071T, *Pseudomonas putida* ATCC 12633T, *Moraxella osloensis* ATCC 19976T, *Acinetobacter haemolyticus* ATCC 17906T, *Acinetobacter baumannii* ATCC 19606T, *Acinetobacter johnsonii* ATCC 17909T, *Acinetobacter lwoffii* ATCC 15309T, *Brevundimonas diminuta* ATCC 11568T, *Moraxella nonliquefaciens* ATCC 19975T, *Pseudomonas aeruginosa* ATCC 10145, *and Burkholderia cepacia* ATCC 25416T. Finally a number of "wild strains" of several bacterial species were also examined. All of these strains had been previously isolated and identified from blood culture from patients at the Freeman Hospital, Newcastle Upon Tyne. This batch of 117 wild strains

was comprised of 9 strains each of *E.coli*, *K.pneumoniae*, *E.cloacae*, *S.marcescens*, *Salmonella* sp. *P.aeruginosa*, *S.epidermidis*, *S.pyogenes*, *E.faecalis*, *E.faecium*, *S.aureus*, *and Proteus mirabilis*. In addition 3 strains of *L.monocytogenes*, 5 of *P.rettgeri* and 1 of *Y.enterocolitica* were used.

Chemicals

3,5-dihydroxy-2-naphthoic acid, *o*-nitrophenol, *o*-nitrophenyl-β-D-xyloside, *o*-nitrophenyl-β-D-glucopyranoside, *o*-nitrophenyl-β-D-galactopyranoside, *o*-nitrophenyl-α-D-galactopyranoside, *o*-(4-nitrophenylphosphoryl)-choline, *o*-nitrophenyl-α-L-fucoside, *o*-nitrophenyl-β-D-fucoside, *o*-nitrophenyl-α-D-glucopyranoside, *o*-nitrophenyl-β-D-glucuronide, *o*-nitrophenyl-N-acetyl-β-D-galactosaminide, L-pyroglutamic acid-*p*-nitroanilide, L-leucine-*p*-nitroanilide, L-phenylalanine-*p*-nitroanilide, γ-glutamyl-*p*-nitroanilide, *p*-nitrophenyl-phosphate, sodium hydrogen phosphate, 4-aminophenol, 4-amino-2,6-dichlorophenol, DEPPD, and acetamidophenol (Paracetamol), were obtained from the Sigma Aldrich Chemical Company Ltd, Poole, UK.

Ammonia copper solution and *o*-cresol were purchased from Cambridge Life Sciences.

Enzyme analysis

β-galactosidase (Grade V1) from *E.coli* and alanyl aminopeptidase was purchased from the Sigma Aldrich Chemical Company Ltd, Poole, UK. The enzymes were prepared by dissolving 1000 units (3.8 mg) in 10 ml of enzyme buffer. Enzyme reaction buffer was prepared by dissolving 0.75g of KCl, 12g NaH_2PO_4 in 800 ml of deionised water. The pH was adjusted to 7.0 using 1 mmol l^{-1} NaOH, and the volume made up to 1 litre.

Media

Columbia agar base and nutrient broth was supplied by Oxoid, Basingstoke.

Substrates

All novel substrates were synthesised by Dr A.L James, University of Northumbria.

Enzyme studies

These were performed using the automated Cobas MIRA analyser, Roche Diagnostics Limited, Lewes.

Detection of staphylocoagulase

Prothrombin (Factor II), TRISMA, t-boc-val-pro-arg-7-amido-4-methylcomarin were obtained from Sigma. Prothrombin was dissolved in water to produce a 1 mg ml^{-1} solution.

A TRISMA/naphthol mixture was prepared by dissolving 3,5-dihydroxy-2-naphthoic acid in 10 ml TRISMA buffer (0.05 mol l^{-1}, pH 9.0) to produce a 5 mmol l^{-1} naphtholic solution. This solution was then filter sterilised. t-boc-val-pro-arg-DMPPD provided by Dr James, proved to be soluble in water and was dissolved in TRISMA/naphthol buffer to produce double-strength solutions of 5 mg ml^{-1}, 3 mg ml^{-1}, 1 mg/ml, 0.5 mg ml^{-1}, 0.1 mg ml^{-1}, 50 µg ml^{-1} and 25 µg ml^{-1}.

Equipment

All compounds were weighed out using a Sartorius 2434 electronic balance; accurate to 0.1 mg. (Sartorius Limited, Epsom, UK). Semi-automatic Gilson pipettes (P200 and P1000) with sterile disposable tips were used in all experiments (Gilson Medical Electronics, Villiers-le-Bel, France). Reaction mixtures were placed into sterile flat bottom microtitre plates (Bibby Sterilin Limited, Aberbargoed, UK) and incubated in a LEEC 37°C shaking incubator (Laboratory and Electrical Engineering Company, Nottingham, UK). A Labtech F1 fluorescence microtitre plate reader was used for assays involving fluorescent substrates.

Enzyme assays

Determination of the absorption spectra of the coloured complexes formed by 4-aminophenol, 4-amino-2,6-dichlorophenol and DEPPD in the presence of *o*-cresol and ammonium copper solution.

Absorption spectra were initially prepared for the coloured reaction products obtained with 4-aminophenol, 4-amino-2,6-dichlorophenol, and DEPPD in reaction with *o*-cresol and ammonium copper solution. This was performed by preparing a 10 mmol l^{-1} solution of each compound by dissolving 14.5 mg of 4-aminophenol, 17.8 mg of the halogenated derivative, and 23.7 mg of DEPPD initially in 200 µl of DMSO, and adding 9.8 ml of sterile reaction buffer. One ml of each dilution and control was added to 1 ml of an equal volume mixture of *o*-cresol and ammonium copper solution. This was prepared by adding an equal volume of each compound (Cambridge Life Sciences) in a plastic universal container. The coloured product, which occurred instantly, was allowed to develop for 2 minutes and absorption spectrum produced over the range 500-700 nm. As a control 1 ml volumes of *o*-cresol and ammonium copper solution were mixed with 1 ml of sterile reaction buffer, this was placed in the reference beam of the instrument and all values obtained were therefore blank

adjusted. Absorbtion spectra were produced for each coloured complex of 4-aminophenol, 4-amino-2,6-dichlorophenol, and DEPPD respectively. Using the peak absorbtion wavelength an attempt was then made to determine the molar absorption coefficient, from which, if the coloured solution obeyed the Beer-Lamberts law (Reed *et al,* 1998) the value could be determined. From these spectra it was clear that the Cobas MIRA analyser could be ued to perform these experiments, since the filters on the instrument closely matched those of the peak absorbances of the generated coloured complexes.

Initially an experiment was performed to produce a molar absorptivity for each coloured complex, at the test wavelengths. These were determined based on the filters present in the instrument, closest to the peak absorbance demonstrated earlier. This was 620 nm for 4-aminophenol and 600 nm for the dichloro phenol derivative, and 620 for the DEPPD complex. The molar absorptivity is the absorbance given by a solution with a concentration of 1 mol l^{-1} of the compound in a light path of 1 cm (Reed *et al.,* 1998). These were determined using a range of dilutions of the 10 mmol l^{-1} stock aminophenols, DEPPD, and for comparison *o*-NP solutions. Briefly 100 µl of each solution was double diluted in a microtitre plate using sterile enzyme buffer. This produced concentrations from 10-0.0012 mmol l^{-1}. A 100 µl volume of *o*-cresol/ammonia copper solution was then added and the colour allowed to develop for 2 minutes. All tests were performed in

triplicate, the blank was 100 µl of *o*-cresol/ammonia copper solution plus 100 µl of enzyme buffer or enzyme buffer only for *o*-NP. Absorbance was determined using the Cobas MIRA analyser at the test wavelengths, and 420 nm for *o*-nitrophenol. Plot could then be generated from blank-adjusted absorbances versus concentration to give the molar absorptivity for each compound by extrapolation from the plot of the concentration giving on absorbance of 1.0.

Determination of the K_m and V_{max} derived from Lineweaver-Burk plots of 4-aminophenyl-β-D-galactoside, and 4-amino-2,6-dichlorophenyl-β-D-galactoside using purified β-galactosidase

It was essential to compare the rates of substrate hydrolysis of these novel chromogens with reference to the most commonly used chromogenic β-galactosidase substrate. Production of Lineweaver-Burk plots and therefore determination of both K_m and V_{max} will indicate the usefulness of the substrates compared with other chromogenic substrates. A comparison was made therefore using 4-aminophenyl-β-D-galactoside, 4-amino-2,6-dichlophenyl-β-D-galactoside and ONPG using purified *E.coli* β-galactosidase. This was performed by the following method. A stock solution of 4 mmol l^{-1} ONPG was prepared by dissolving 60 mg of ONPG in 50 ml of sterile reaction buffer. The solution was stored in an amber bottle until use. A stock solution of the enzyme β-galactosidase was prepared by

dissolving 1000 units in 10 ml of sterile de-ionised water. One unit of enzyme is known to hydrolyse 1 μmol of *o*-nitrophenyl-β-D-galactoside to *o*-nitrophenyl and D-galactose per minute at pH 7.3 at 37°C. A cuvette containing 95 μl of ONPG solution was placed into the Cobas MIRA analyser and mixed with 5 μl of a 1:10 dilution of β-galactosidase stock solution. Absorbance readings at 420 nm were taken at 24-second intervals for 6 minutes. As a control 95 μl of ONPG plus 5 μl of sterile de-ionised water was used. All absorbance readings produced were blank adjusted. A graph of absorbance versus time was then produced. The experiment was then repeated for a range of ONPG concentrations. The 4 mmol l^{-1} stock solution was double diluted to 1/256 in reaction buffer, enzyme was added and the absorbance determined as described previously. These values allowed the production of Lineweaver-Burk plots and determination of K_m.

The experiment was repeated for the substrates 4-aminophenyl-β-D-galactoside and 4-amino-2,6-dichlorophenyl-β-D-galactoside by preparing stock 4mmol l^{-1} solutions of each. These were prepared by adding 10.8 mg and 13.5 mg respectively to 10 ml of reaction buffer and warming gently to dissolve. Double dilutions of each substrate were prepared to a dilution of 1/256. A 95 μl aliquot of the undiluted stock or each dilution was placed into a cuvette. The reaction was then started by adding 5 μl of a 1:10 dilution of β-galactosidase. At 60-second intervals, a 100 μl aliquot of

mixture containing an equal volume of both ammonium copper and *o*-cresol solutions was added. This solution also stopped the action of β-galactosidase due to the high concentration of Cu^{2+} ions. After 2 minutes the colour of each cuvette containing 4-aminophenol-β-D-galactoside or the dichlorophenol substrate was measured at an absorbance of 620 nm for the 4-aminophenol substrate, and for the 580 nm for the halogenated substrate, using the automated Cobas MIRA analyser instrument. This produced a series of fixed-point determinations, which was used to produce Lineweaver-Burk plots, and the K_m of the reaction determined. Both blank and test absorbances were performed in triplicate.

Evaluation of substrates in liquid media for the detection of a range of bacterial hydrolyases.

Each substrate was carefully weighed out in a glass universal container and dissolved in 1 ml of 0.1mmol l^{-1} NaOH and made up to volume in 9 ml of API buffer, and the pH re-adjusted to 7.2. The solution was filter sterilised using a 0.45 μm Millipore filter. Each substrate was prepared at a concentration of 5 mmol l^{-1}. Similarly a 10 mmol l^{-1} solution of 3,5-dihydroxy-2-naphthoic acid was prepared by dissolving 5.1 mg in 1 ml of 0.1 mmol l^{-1} NaOH and making up to volume in 9 ml of API buffer, the pH re-adjusted to 7.2, and the solution filter sterilised.

A suspension of each organism, freshly harvested from an overnight subculture was made in sterile API buffer. The suspension was prepared equal to a McFarland standard of 1.0 (Approx 2 x 10^8 cfu ml^{-1}) using an API densimat. A 50 µl volume of each organism suspension was then added to the substrate/naphthol well. As a control a 50 µl volume of each substrate and 50 µl of naphthol was used. The plate was placed into a shaking incubator and examined visually after four hours for the production of a coloured reaction product.

As a control, the *p*-nitroanilide or nitrophenolic equivalent of each substrate was tested in parallel. Each *p*-nitroanilide substrate was prepared as a 5 mmol l^{-1} solution by dissolving initially in 400 µl of dimethyl sulphoxide (DMSO) and making up to volume in 9 ml of API buffer, and re-adjusting the pH to 7.4. The solution was then sterilised by membrane filtration. Nitrophenolic substrates were prepared at a concentration of 5 mmol l^{-1} by dissolving in 10 ml of API buffer. The solution was warmed gently to aid dissolving. The pH was re-adjusted to 7.2 and the solution was sterilised by membrane filtration.

A 50 µl volume of each *p*-nitroanilide or nitrophenolic solution was added to each well of a microtitre plate. A 50 µl volume of each organism suspension was then added. As a control, 50 µl volumes of both phosphate buffer and substrate solution were added to a single well of the microtitre

plate. Each assay was performed in duplicate in a separate microtitre plate. The plates were then covered with parafilm and placed into a shaking incubator, and examined visually after four hours for the production of the characteristic yellow reaction product.

For the detection of staphylocoagulase the following method was used:- Thirteen strains of staphylococci, and a negative control strain, *E.faecalis*, were freshly sub-cultured on Columbia blood agar and suspended in brain heart infusion broth to a turbidity equivalent to a McFarland standard of 4.0 (4 x strength inoculum). The strains used were *S.aureus* (12), *S.epidermidis* (1) and *E.faecalis* (1). The assays were performed in duplicate in two separate microtitre plates. Briefly, fifteen wells of each microtitre plate was inoculated with 25 µl of prothrombin solution, followed by 25 µl of each organism suspension. Well 15 served as an organism-free control. The plate was incubated at 37°C for 40 minutes. After incubation, 50 µl of 5 mg ml^{-1} of t-boc-val-pro-arg-DMPPD was added to each well (final concentration 2.5 mg l^{-1}). The tests were repeated using a range of concentrations of substrate from 5 - 0.25 mg l^{-1} substrate. Wells of each plate were monitored visually over 18 hours for colour development. As a control, the substrate t-boc-val-pro-arg-7-AMC was prepared at a concentration of 0.5 mg ml^{-1} (final concentration 0.25 mg ml^{-1}) and examined under the same experimental conditions as for the test substrate. Plates containing the fluorescent substrate were incubated for 2 hours and examined for

fluorescence (excitation 365 nm, emission 440 nm) every 30 minutes.

Results

The absorbance spectra for the coloured complex generated by the coupling of 4-aminophenol, 4-amino-2,6-dichlorophenol and DEPPD with *o*-cresol and ammonium copper solution are shown in Figures 3.6 to 3.8. It was interesting to note that the peak absorbance for aminophenol upon reaction with *o*-cresol and ammonium copper solution was 615 nm. This is in agreement with the manufacturers published data (Product information sheet, Cambridge Life Sciences). However it was interesting to note that the absorbance peak of the halogenated compound which has not been previously determined was lower at 584 nm. Presumably, chlorine substitutions at positions 2,6 of the aminophenol ring, shifts the absorbance toward the green waveband of the spectrum. It would be interesting to perform the spectrum for the di-bromo derivative in the future to see if the more electron-dense halogen substitute results in a further shift towards shorter wavelengths. The peak absorbance for the DEPPD/cresol complex was 650 nm. Using these results it was decided to perform all fixed-point enzyme assays using substrates of 4-aminophenol, 4-amino-2,6-dichlorophenol and DEPPD in the Cobas MIRA analyser at 620, 580 and 620 nm respectively, since the peaks are sufficiently broad for this to be effective.

Figure 3.6. Absorption spectrum produced by a 10 mmol l^{-1} solution of DEPPD/*o*-cresol/ammonium copper complex

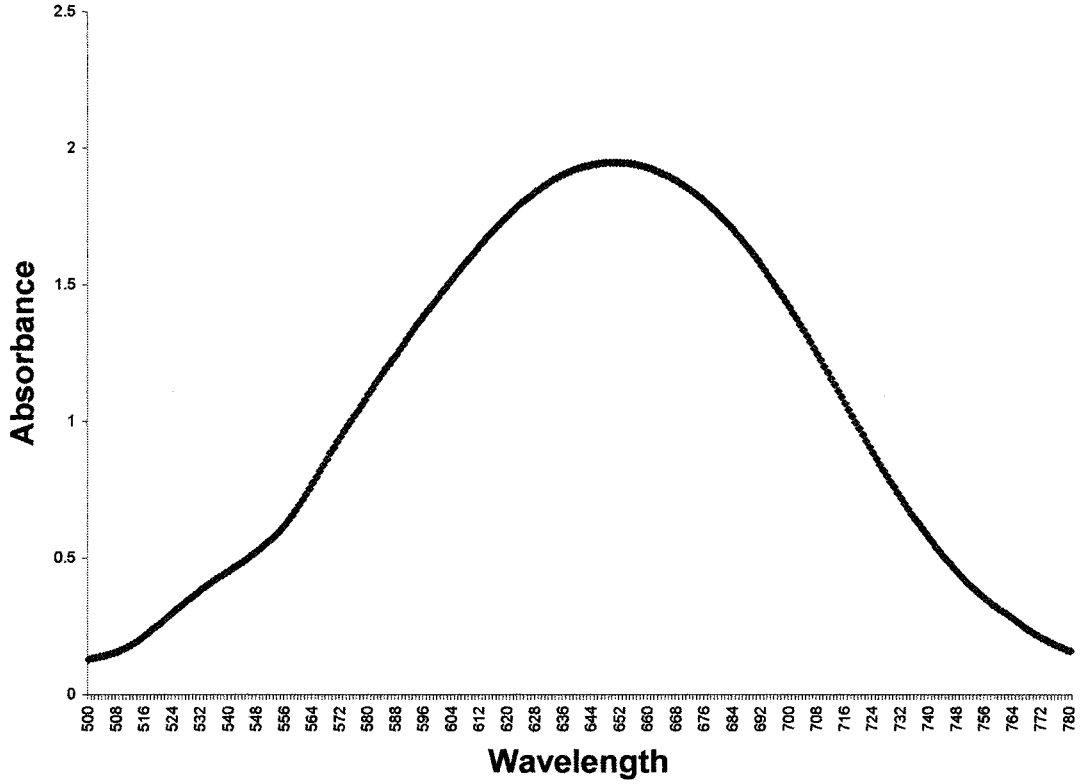


Figure 3.7. Absorbance spectrum produced by a 10 mmol l^{-1} solution of 4-aminophenol/*o*-cresol/ammonium copper complex.

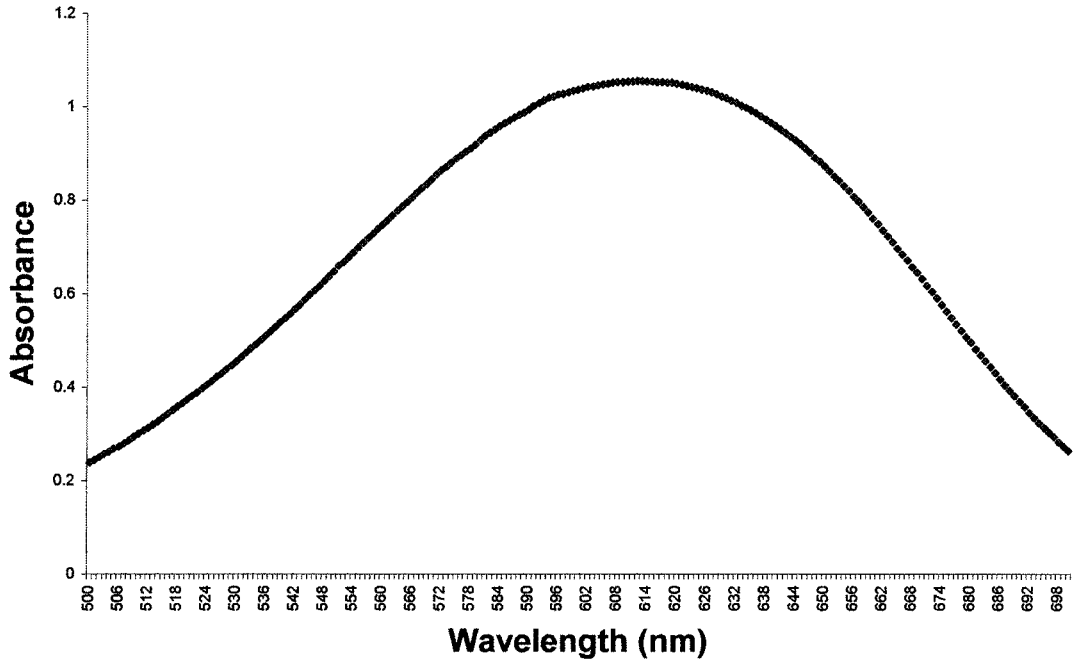
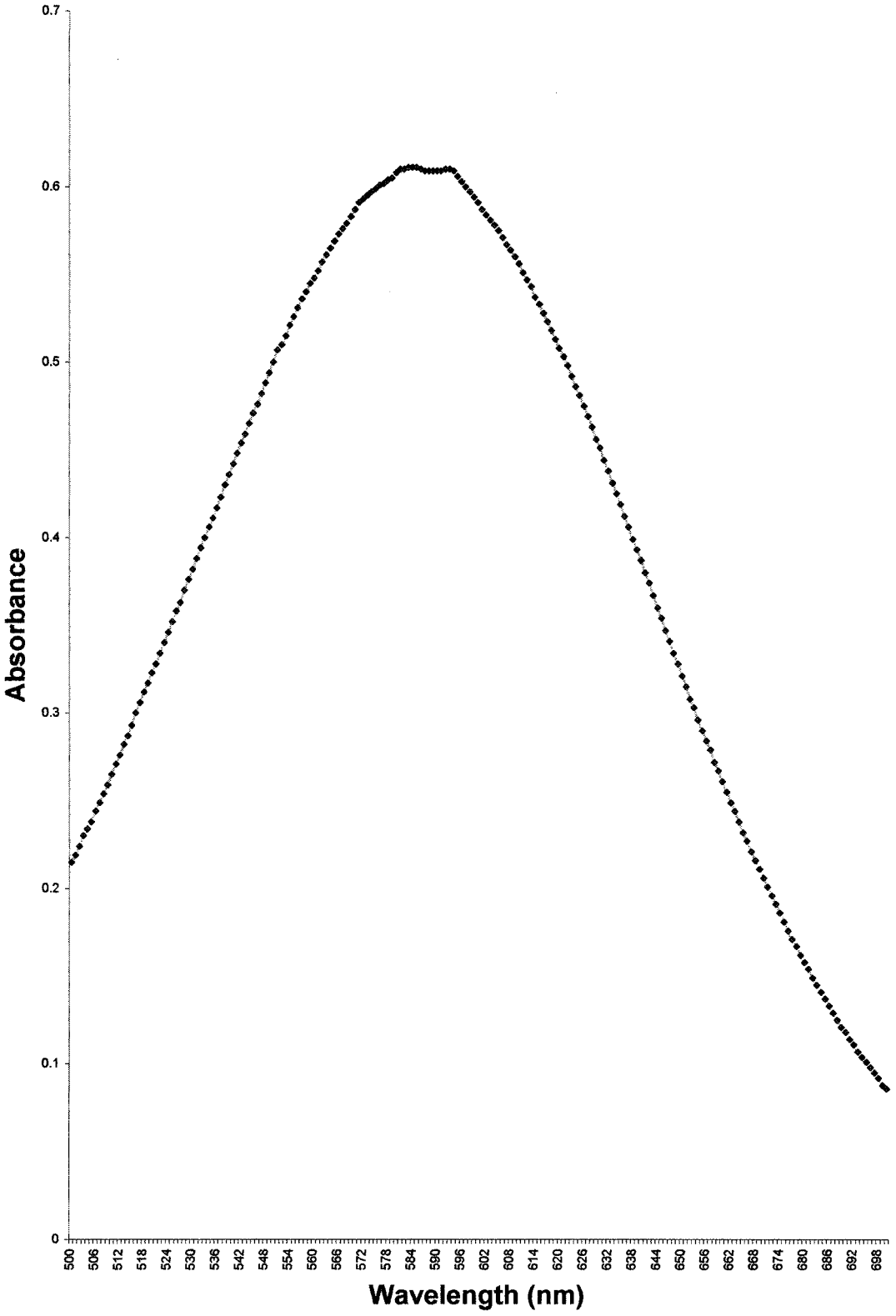


Figure 3.8. Absorbance spectrum produced by a 10 mmol/l solution of 4-amino-2,6-dichlorophenol/*o*-cresol/ammonium copper complex.



It was clear from the results of the experiment to determine the molar absorbtivity of 4-aminophenol, 4-amino-2,6-dichlorophenol, DEPPD and o-nitrophenol, was that the coloured complex did not obey the Beer-Lambert law. The plot of concentration versus absorbance was non linear and as such the value could not be determined. A mathematical transformation of the absorbance and concentration was performed to produce a linear relationship. This was done by plotting absorbance versus concentration for each of the test compounds on a logarithmic scale. The plots of these are shown in figures 3.9 to 3.12. These logarithmic plots were then used to determine K_m and V_{max} for each of the test compounds. This was performed by dermination of the log of the absorbance change per minute for each substrate concentration and extrapolation from the plot to produce the concentration. These values were input into Enzpack computer software and a Lineweaver-Burk plot produced (Figures 3.13 - 3.15). From this the K_m and V_{max} values were determined. These values are shown in Table 3.4.

Figure 3.9. Plot of absorbance v concentration for the coloured complex formed between 4-aminophenol and o-cresol/ammonia copper complex on a logarithmic scale

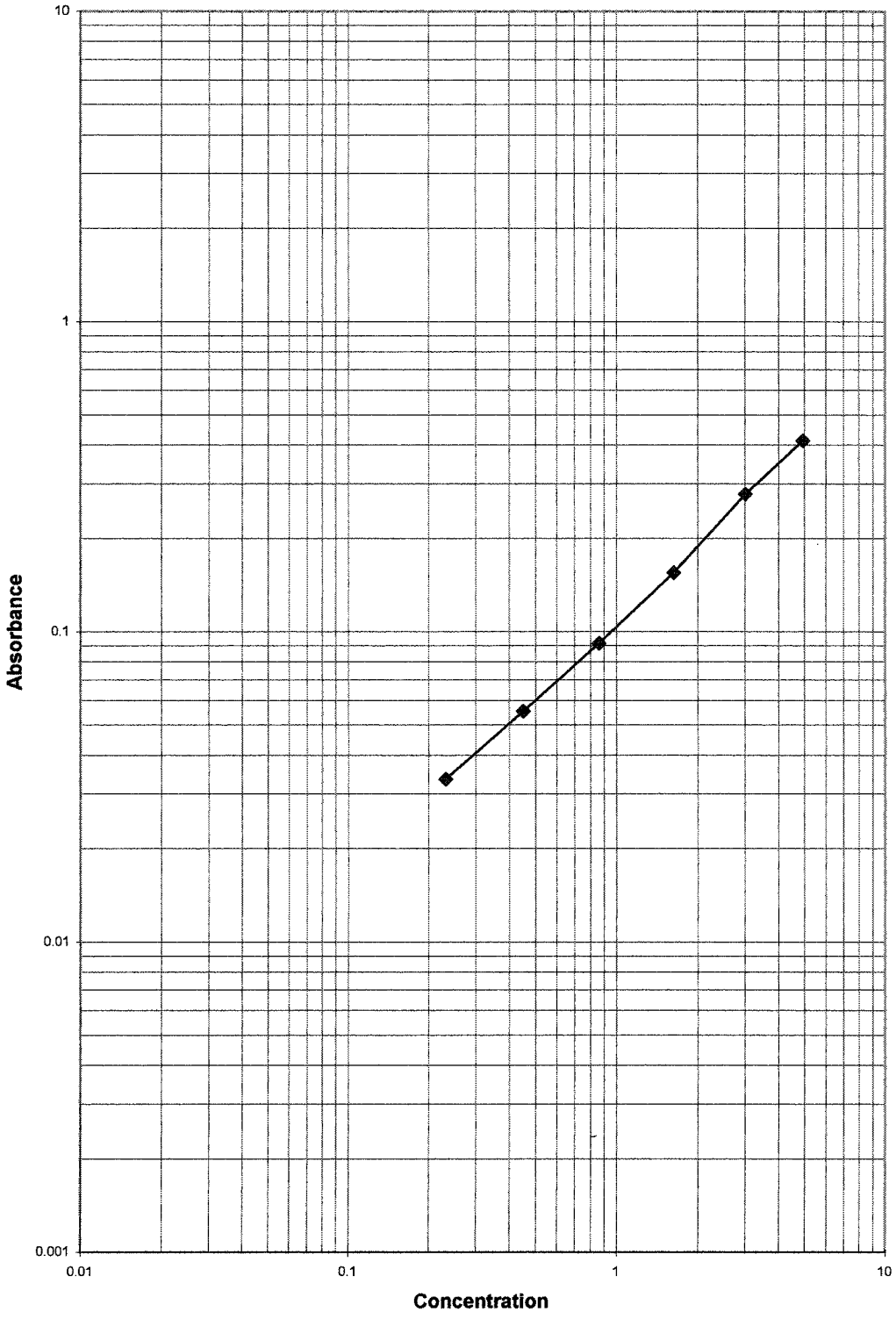


Figure 3.10. Plot of absorbance v concentration for the coloured complex formed between DEPPD and o-cresol/ammonium copper complex on a logarithmic scale

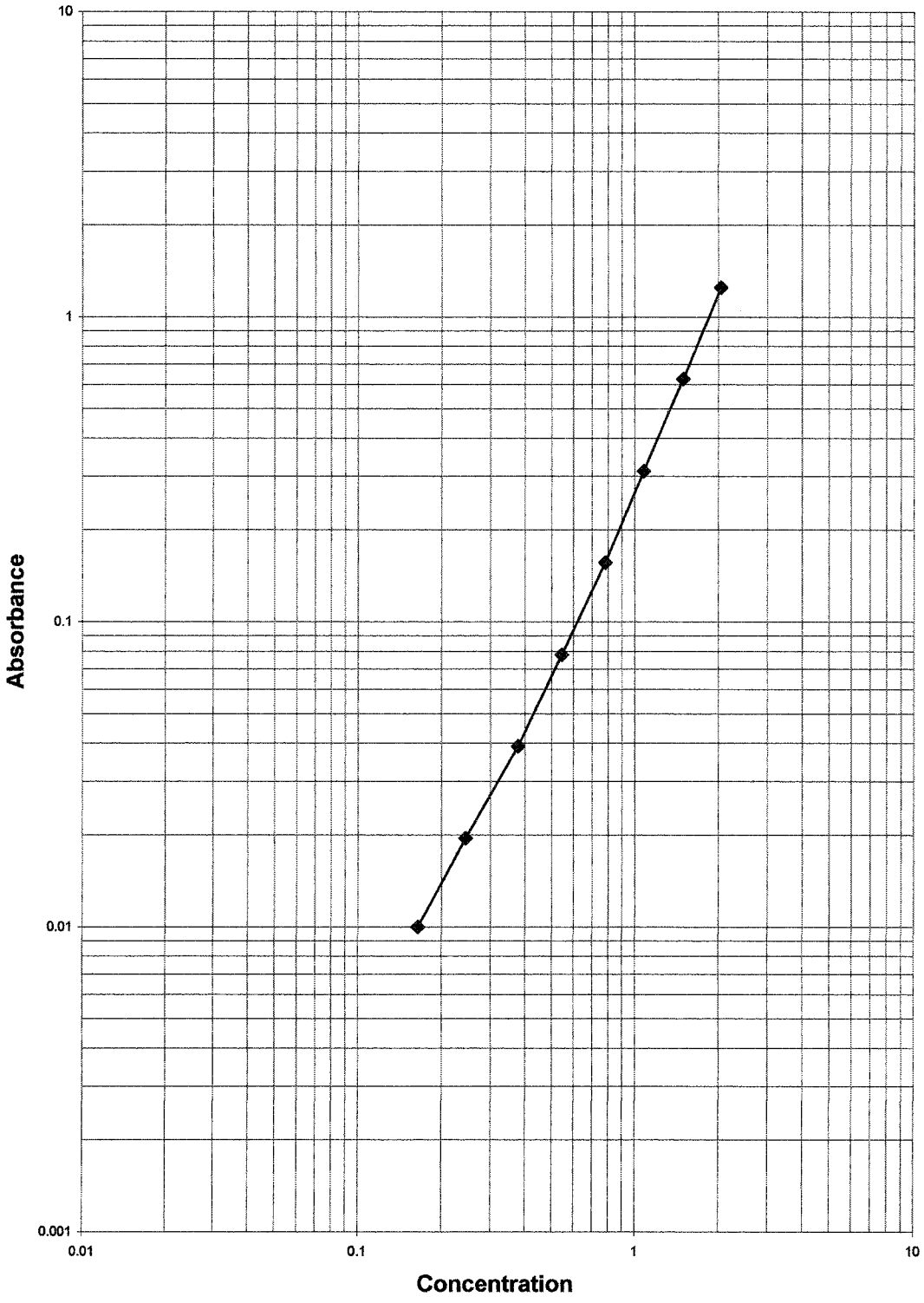


Figure 3.11. Plot of absorbance v concentration for the coloured complex formed between 4-amino-2,6-dichlorophenol and o-cresol/ammonium copper solution on a logarithmic scale

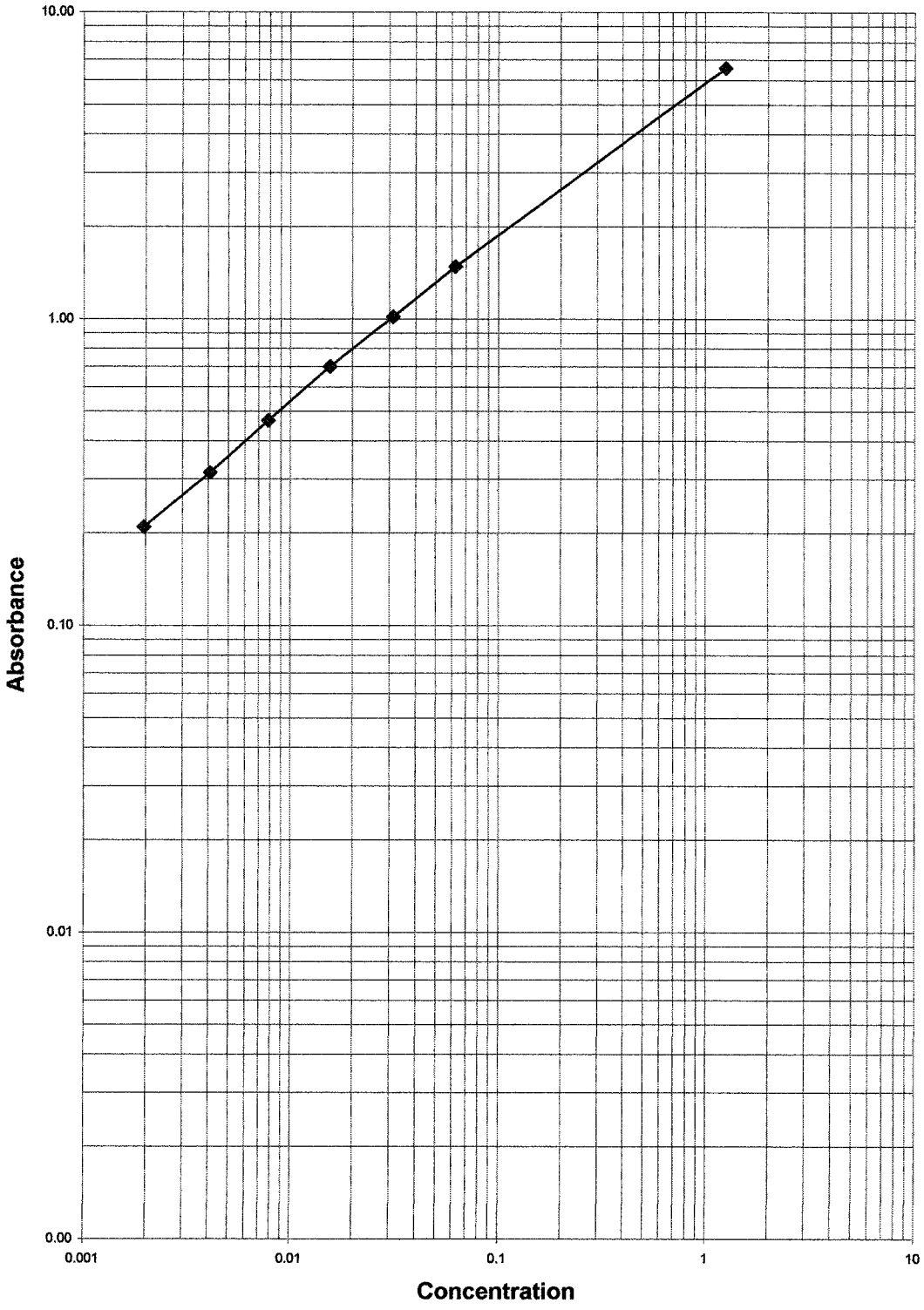


Figure 3.12. Plot of absorbance v concentration for *o*-nitrophenol on a logarithmic scale

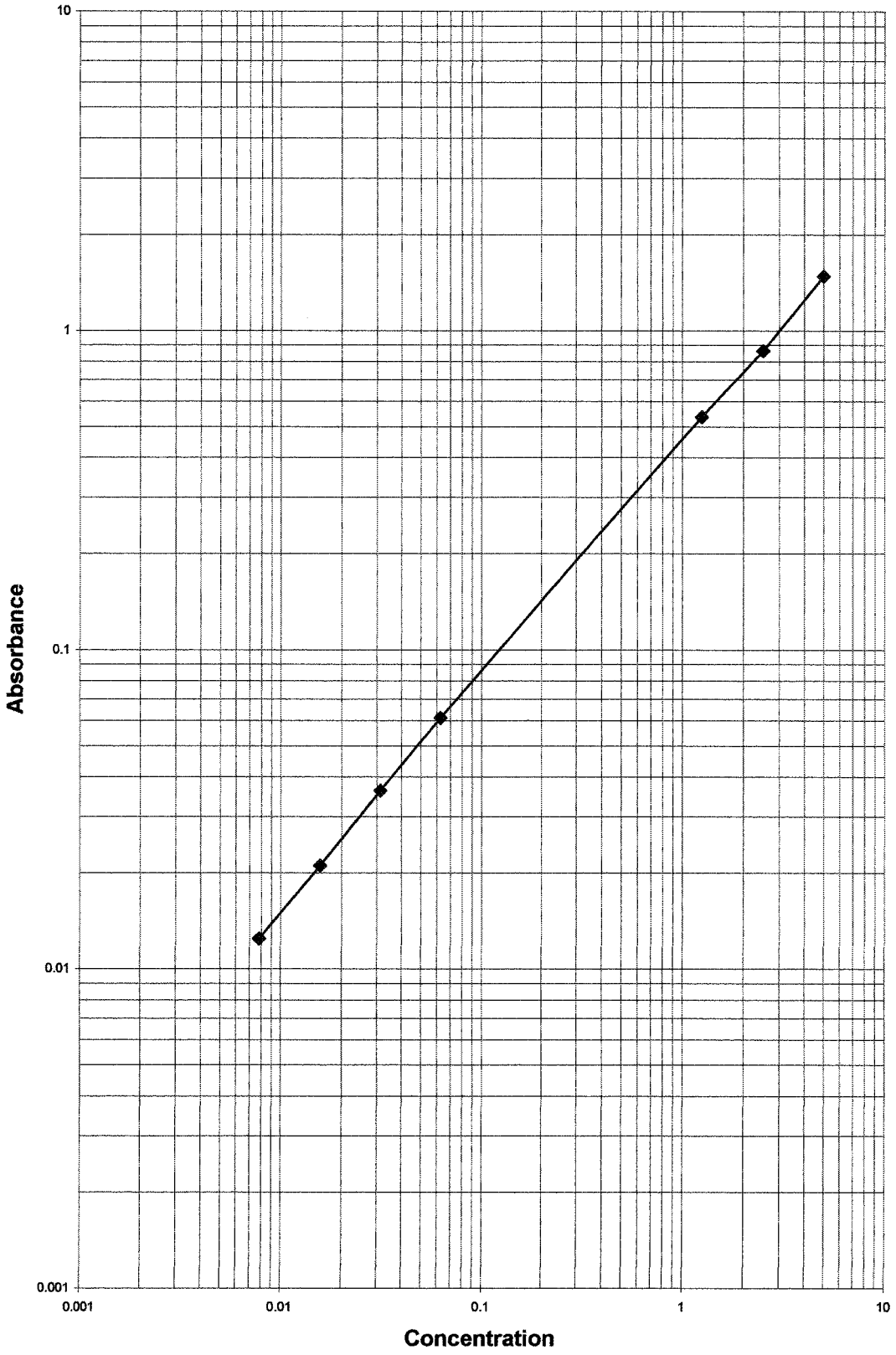


Figure 3.13 Lineweaver-Burk plot for hydrolysis of ONPG by β-galactosidase.

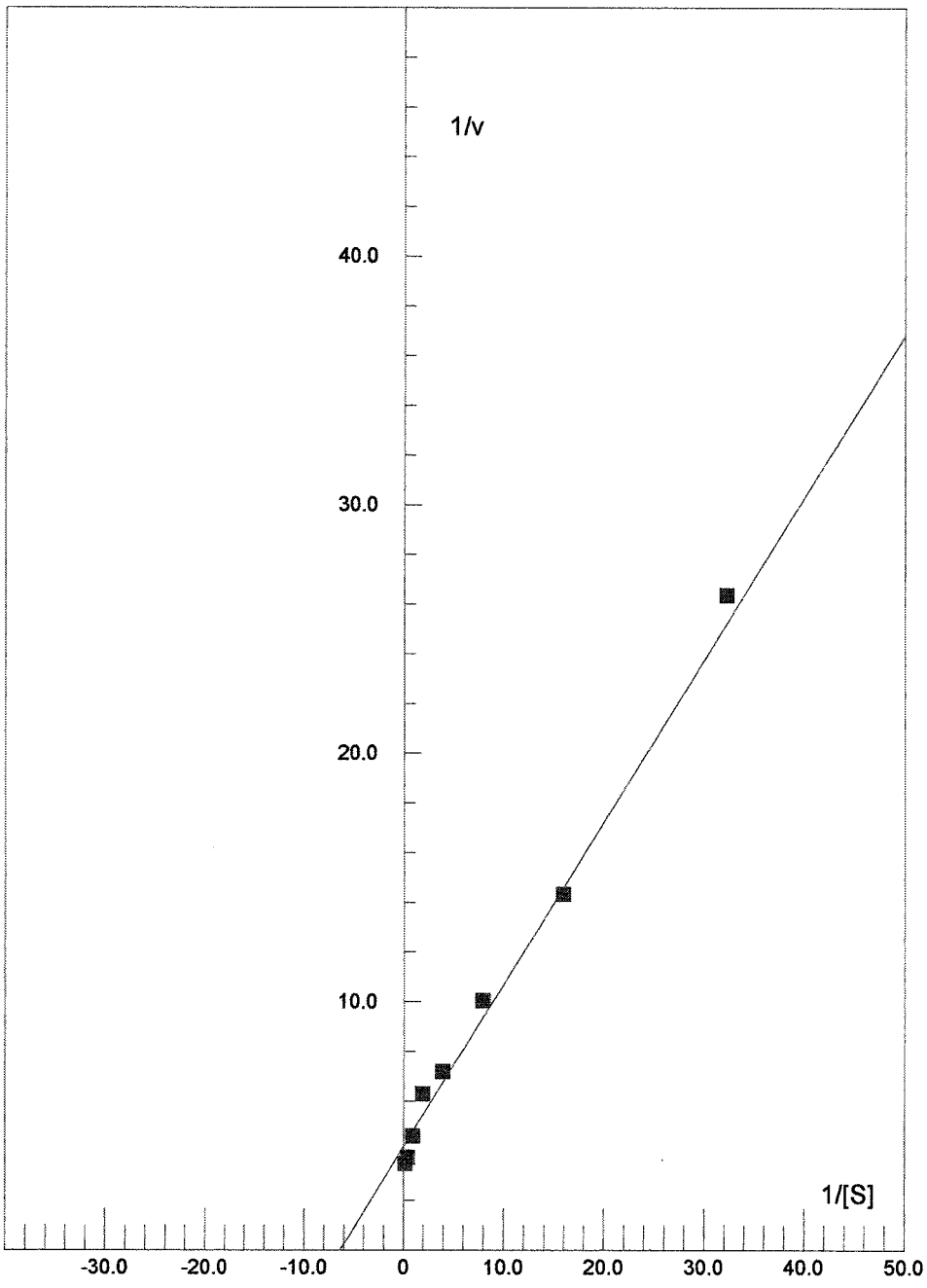


Figure 3.14 Lineweaver-Burk plot for hydrolysis of 4-aminophenyl-β-D-galactoside by β-galactosidase.

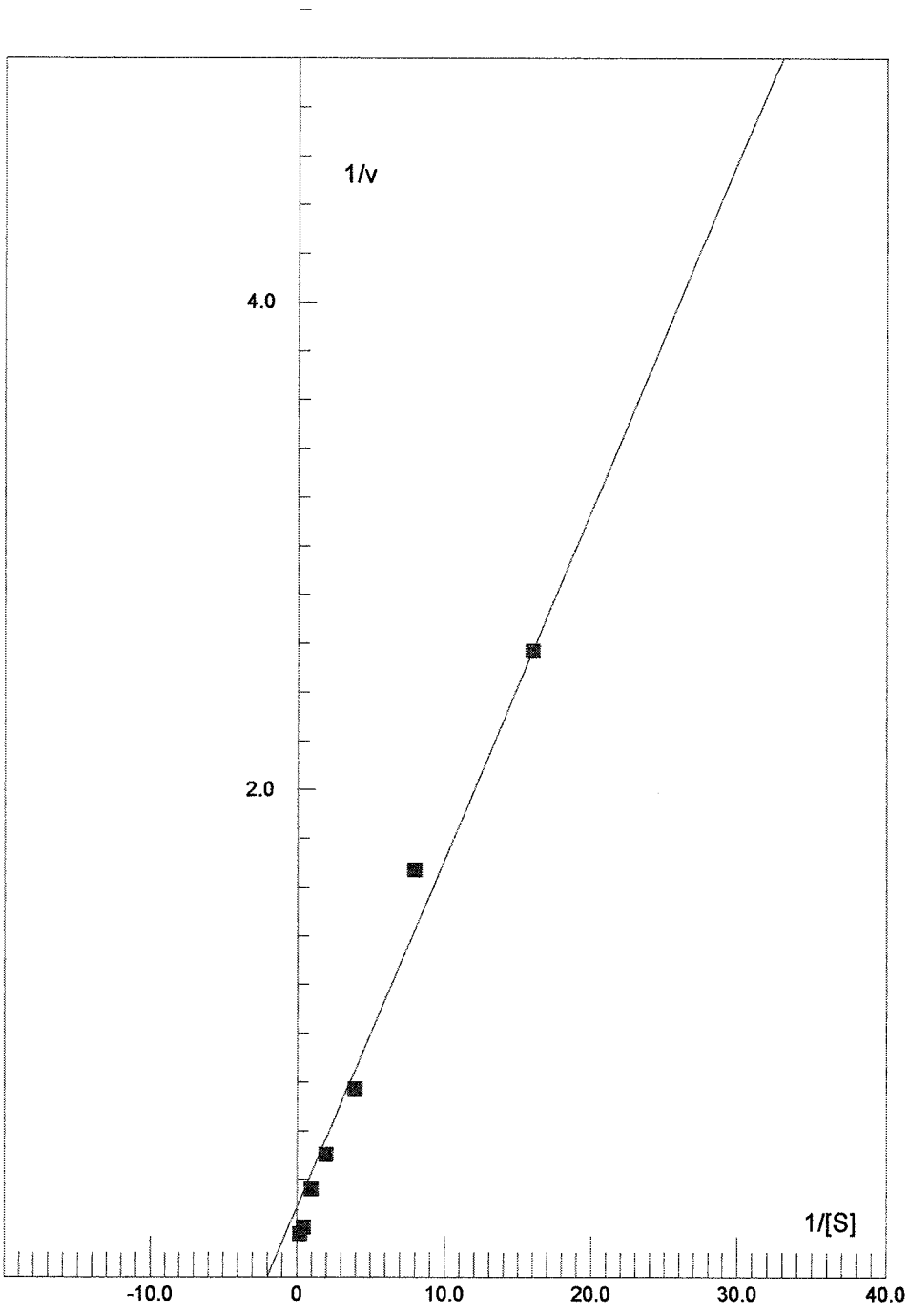
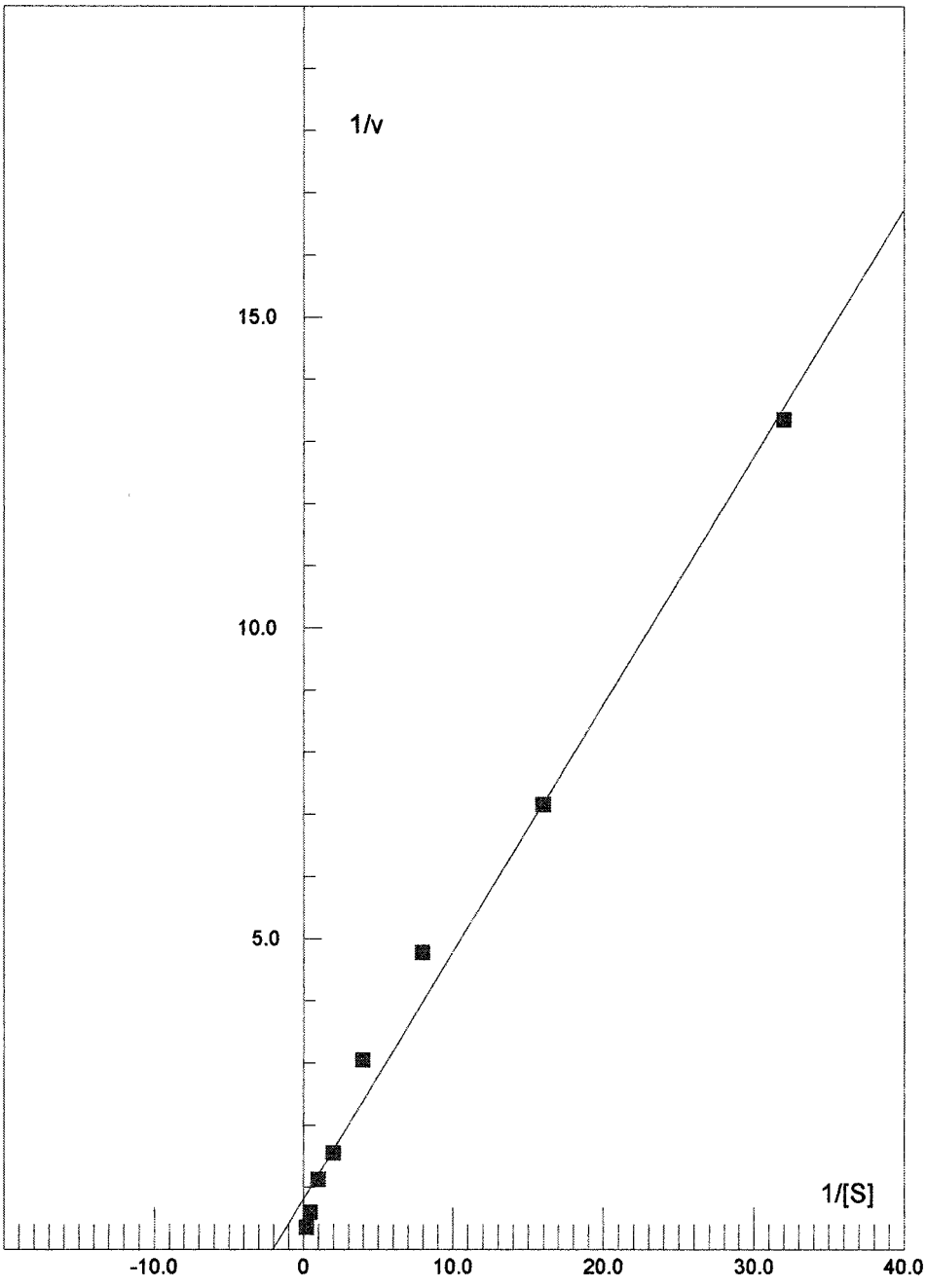


Figure 3.15 Lineweaver-Burk plot for hydrolysis of 2,6-dichloro-4-aminophenyl-β-D-galactoside by β-galactosidase.



It was interesting that the absorbance for the complex generated from the halogenated derivative was significantly less than that observed with simple 4-aminophenol. It is possible that this is due to differences in affinity in the coupling between the core compound and *o*-cresol solution, due to the di-substituted halogen atoms, and as such a less-coloured complex is formed. The 4-aminophenol complex produced the strongest absorbance of all of the test compounds.

Table 3.1 shows the blank adjusted absorbance values produced by *o*-nitrophenol from the hydrolysis of a range of ONPG concentrations by β-galactosidase at 420 nm. Tables 3.2 and 3.3 show the blank adjusted absorbance values produced by the 4-aminophenol and 4-amino-2,6-dichlorophenol/*o*-cresol complexes formed upon hydrolysis of the β-D-galactoside substrates of both by β-galactosidase. The values for the initial reaction velocity of each test substrate versus concentration was calculated by producing a graph of concentration over time and drawing a tangent. Values are expressed as absorbance change per minute. The derived values from the Lineweaver-Burk plots for both K_m and V_{max} for each test substrate are shown in Table 3.4.

Table 3.1. Blank adjusted absorbances (420 nm) of released o-nitrophenol from hydrolysis of ONPG by beta-galactosidase

	Concentration (mmol l^{-1})								
	4.00	**2.00**	**1.00**	**0.50**	**0.25**	**0.13**	**0.06**	**0.03**	**0.02**
Time (sec)									
0	0.08	0.06	0.05	0.05	0.04	0.04	0.04	0.04	0.04
24	0.22	0.18	0.15	0.13	0.11	0.09	0.07	0.06	0.05
48	0.56	0.47	0.41	0.34	0.26	0.18	0.12	0.08	0.06
72	0.90	0.79	0.68	0.53	0.35	0.21	0.13	0.08	0.06
96	1.22	1.11	0.94	0.66	0.38	0.22	0.13	0.09	0.06
120	1.53	1.43	1.15	0.72	0.39	0.22	0.13	0.09	0.06
144	1.82	1.76	1.31	0.73	0.39	0.22	0.13	0.09	0.06
168	2.09	2.06	1.37	0.74	0.39	0.22	0.13	0.09	0.06
192	2.34	2.33	1.39	0.73	0.39	0.22	0.13	0.09	0.06
216	2.57	2.53	1.39	0.73	0.39	0.22	0.13	0.09	0.06
240	2.77	2.64	1.39	0.73	0.39	0.22	0.13	0.09	0.06
264	2.96	2.70	1.38	0.73	0.39	0.22	0.13	0.09	0.06
288	3.10	2.72	1.39	0.73	0.39	0.22	0.13	0.09	0.06
312	3.28	2.72	1.39	0.73	0.39	0.22	0.13	0.09	0.06
336	3.43	2.72	1.38	0.73	0.39	0.22	0.13	0.09	0.06
360	3.52	2.69	1.38	0.73	0.39	0.22	0.13	0.09	0.06
384	3.58	2.70	1.38	0.73	0.39	0.22	0.13	0.09	0.06

Table 3.2 Blank adjusted absorbances (620 nm) produced by the 4-aminophenol/o-cresol complex by beta-galactosidase

	Concentration (mmol l^{-1})								
	4.00	**2.00**	**1.00**	**0.50**	**0.25**	**0.13**	**0.06**	**0.03**	**0.02**
Time (sec)									
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
24	0.22	0.13	0.07	0.05	0.03	0.01	0.01	0.01	0.00
48	0.48	0.26	0.13	0.10	0.05	0.04	0.03	0.02	0.01
72	0.71	0.40	0.20	0.23	0.10	0.06	0.05	0.03	0.01
96	0.98	0.61	0.31	0.40	0.20	0.08	0.06	0.03	0.02
120	1.21	0.84	0.42	0.51	0.29	0.12	0.07	0.03	0.02
144	1.54	1.12	0.56	0.54	0.30	0.14	0.07	0.03	0.02
168	1.89	1.44	0.75	0.56	0.30	0.13	0.07	0.03	0.02
192	2.18	1.79	0.94	0.56	0.30	0.14	0.07	0.03	0.02
216	2.51	2.16	1.08	0.56	0.30	0.13	0.07	0.03	0.02
240	2.83	2.23	1.11	0.56	0.30	0.14	0.07	0.03	0.02
264	3.10	2.24	1.09	0.56	0.30	0.14	0.07	0.03	0.02
288	3.32	2.25	1.09	0.56	0.30	0.14	0.07	0.03	0.02
312	3.69	2.27	1.10	0.56	0.30	0.14	0.07	0.03	0.02
336	3.96	2.24	1.08	0.56	0.30	0.14	0.07	0.03	0.02
360	4.11	2.24	1.10	0.55	0.30	0.14	0.07	0.03	0.02
384	4.10	2.24	1.09	0.57	0.30	0.14	0.07	0.03	0.02

Table 3.3. Blank adjusted absorbances (584 nm) produced by the 4-amino-2,6-dichlorophenol/o-cresol complex by beta-galactosidase

	Concentration (mmol l^{-1})								
	4.00	**2.00**	**1.00**	**0.50**	**0.25**	**0.13**	**0.06**	**0.03**	**0.02**
Time (sec)									
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
24	0.210	0.002	0.001	0.001	0.002	0.002	0.003	0.000	0.000
48	0.510	0.008	0.006	0.002	0.003	0.004	0.004	0.002	0.001
72	0.830	0.190	0.041	0.024	0.013	0.008	0.007	0.006	0.004
96	1.150	0.360	0.098	0.075	0.032	0.024	0.019	0.013	0.010
120	1.430	0.570	0.210	0.135	0.096	0.069	0.046	0.036	0.020
144	1.780	0.830	0.330	0.220	0.183	0.121	0.063	0.036	0.023
168	2.100	1.010	0.500	0.390	0.230	0.120	0.064	0.037	0.023
192	2.410	1.290	0.710	0.490	0.240	0.120	0.065	0.034	0.023
216	2.790	1.540	0.900	0.500	0.250	0.124	0.064	0.035	0.025
240	3.100	1.880	0.950	0.500	0.250	0.120	0.065	0.036	0.026
264	3.410	1.950	0.950	0.500	0.260	0.125	0.065	0.036	0.024
288	3.700	1.950	0.950	0.500	0.250	0.120	0.064	0.037	0.023
312	3.720	1.950	0.960	0.490	0.240	0.125	0.062	0.036	0.025
336	3.740	1.930	0.970	0.500	0.250	0.120	0.065	0.039	0.023
360	3.710	1.950	0.970	0.500	0.240	0.122	0.064	0.037	0.022
384	3.720	1.960	0.970	0.510	0.250	0.121	0.064	0.038	0.022

Table 3.4 Values of K_m and V_{max} for each test substrate.

Substrate	K_m (mmol l^{-1})	V_{max} (mmol/l/min $^{-1}$)
ONPG	0.12	0.12
4-aminophenyl-β-D-galactoside	0.5	3.49
4-amino-2,6-dichlorophenyl-β-D-galactoside	0.49	1.24

This data in table 3.4 can be used to compare the hydrolysis of the different substrates. Since K_m is a measure of the affinity of the substrate for the enzyme, the substrate with the lowest K_m has the highest affinity for the enzyme β-galactosidase used in these experiments. From these results the substrate ONPG has the highest affinity for the enzyme, more so than either of the novel test substrates. V_{max}, which is the maximal rate at which the enzyme catalyses a reaction, is derived from the Lineweaver-Burk plot, and expressed as the amount of product formed per minute. This experiment shows that ONPG, which has the lowest K_m value, also has the lowest V_{max} value (0.12 mmol l^{-1}) the conversion of the ES complex to E + P as measured by V_{max} is the poorest, nearly 30-fold less than the simple 4-aminophenol substrate.

V_{max} can also be used to provide the turnover number (K_{cat}). This is the number of substrate molecules transformed to product by one enzyme molecule per unit time, and is calculated from Equation 3.4:-

$K_{cat} = V_{max}/[E]_T$ where $[E]_T$ is the concentration of enzyme (mol l^{-1})

Equation 3.4. Equation for the calculation of turnover number derived from V_{max}.

The molecular weight of the enzyme is known to be 116.5 KDa and as such the final concentration of the enzyme can be calculated. Initially, 3.8 mg were dissolved in 10 ml of enzyme buffer, and 5 µl used, the final enzyme concentration was calculated as 1.63×10^{-8} mol l^{-1}. The equation for the calculation of K_{cat} for ONPG can be represented as V_{max} (0.12 mmol/l^{-1}/min^{-1}) first converted to mol/l^{-1}/s^{-1} = 2×10^{-6} mol/l^{-1}/s^{-1} then divided by the enzyme concentration to achieve a value for K_{cat}, therefore, 2×10^{-6} mol/l^{-1}/s^{-1} /1.63×10^{-8} mol l^{-1} = 122.6 s^{-1}. Repeating this calculation for 4-aminophenyl-β-D-galactoside the K_{cat} is calculated as 5.8×10^{-5} mol/l^{-1}/s^{-1}/1.63×10^{-8} mol l^{-1} = 3558 s^{-1}, and for 4-amino-2,6-dichlorophenyl-β-D-galactoside as 2×10^{-5} mol/l^{-1}/s^{-1} /1.63×10^{-8} mol l^{-1} = 1227 s^{-1}.

The K_{cat} value is useful since it measures the efficiency of the enzyme, and the higher the K_{cat} value the "better" the enzyme hydrolyses the substrate. Thus the highest K_{cat} value was achieved for the simple 4-aminophenyl substrate followed by the dichloro derivative and finally ONPG. The reciprocal of K_{cat} is the time required by an enzyme molecule to "turn over" one substrate molecule. Calculated reciprocal values for ONPG, 4-

aminophenyl-β-D-galactoside and 4-amino-2,6-dichlorophenyl-β-D-galactoside are 8.2×10^{-3}, 2.8×10^{-4} and 8.1×10^{-4} seconds respectivly. These values reflect V_{max} in that the β-galactosidase "turns over" more 4-aminophenol-β-galactoside molecules faster than the other 2 substrates. The nitrophenolic substrate as expected shows the slowest rate of "turn over". The ratio of K_{cat}/K_m is regarded as a direct measure of enzyme efficiency for a particular substrate. For each substrate this value for enzyme efficiency is calculated for the substrate ONPG as $122.6/0.12 \times 10^{-3} = 1.02 \times 10^6$ mol/l^{-1}/s^{-1} , for 4-aminophenyl-β-D-galactoside as $3558.2/0.5 \times 10^{-3} = 7.11 \times 10^6$ mol/l/s^{-1} and the dichloro substrate as $1227/0.49 \times 10^{-3} = 2.5 \times 10^6$ mol/l^{-1}/ s^{-1}.

These values are published for a wide variety of enzymes, the higher the ratio K_{cat}/K_m the more efficient the enzyme is towards the substrate. For this experiment 4-aminophenyl-β-D-galactoside has the highest efficiency for the enzyme β-galactosidase derived from *E.coli*. These values will be important in determining the usefulness of these novel substrates for the rapid detection of bacterial hydrolyases. These substrates were examined initially in liquid media and then solid media. Although these results would suggest that the 4-aminophenol substrate would be hydrolysed faster than ONPG by *E.coli* β-galactosidase, due to higher K_{cat}/K_m values, whether this will extrapolate to other glyosidases will be shown later in this chapter

based on studies of the substrates in liquid media. Since the K_{cat}/K_m value for 4-aminophenyl-β-D-galactoside is high, by comparison to ONPG, this would suggest the substrate would produce excellent results in liquid media. It would also be expected that the chlorinated substrates would also perform well since the K_{cat}/K_m values are higher than those obtained with ONPG. Practically however, over a typical time period of 2-4 hours the differences in enzyme efficency may be difficult to determine visually, especially if a high biomass is used. Moreover when incorporated into agar, incubation periods of 18-24 hours would be expected to produced enough detectable reaction/coupling product for the colonies to be easily visualised. The enzyme used in these experiments is from *E.coli* and enzymes derived from other bacterial or mammalian sources may produce different results, and this may also be reflected not only in whether a specific organism produces a positive reaction, but also on how quickly the results are determined.

GLYCOSIDASE SUBSTRATES

Evaluation of 4-aminophenyl-β -D-galactoside (4-amino-GAL), 4-amino-2,6-dichlorophenyl-β-D-galactoside (DCAP-GAL), 4-aminophenyl-α-D-galactoside (4-amino-AGAL), ONPG, and *o*-nitrophenyl-α-D-galactoside (ONPAGAL) in liquid format.

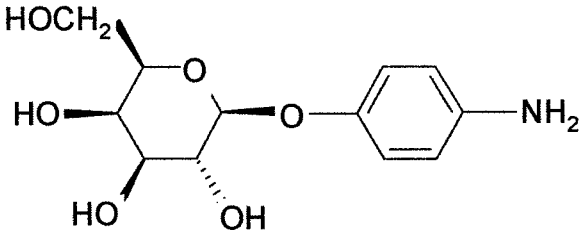


Figure 3.16 Chemical structure of 4-aminophenyl-β-D-galactoside

The results of this experiment are summarised in Table 3.5. In addition Figure 3.17 shows the reaction of 15 NCTC strains in the presence of 3 substrates for the detection of β-galactosidase activity. The substrate, 4-amino-GAL (Figure 3.16) appeared to work well and produced a red/orange coloration with ß-galactosidase producing strains. For strains of *Enterobacteriaceae,* correlation with the *p*-nitrophenyl derivative was 100%, although 2 wild strains of *E.cloacae* required overnight incubation to produce a positive result with the aminophenyl derivative. These strains were rapidly reactive in the wells containing the nitrophenolic substrate. The halogenated substrate, produced results in complete agreement with

those obtained with ONPG, and the typical blue-reaction product was generally easier to read visually than that obtained with either ONPG, or the non-halogenated substrate. All *E.cloacae* strains reacted in 4 hours using the halogenated substrate.

The α-galactoside substrate worked well and again there was excellent correlation with *o*-nitrophenyl-α-D-galactoside (ONPAGAL) over four hours of incubation (Table 3.5). Despite the weak reaction observed with *E.cloacae* using 4-amino-GAL overall both novel substrates produced strong reactions, and clearly would be useful for the detection of β-galactosidase activity.

Table 3.5 Reaction of 132 bacterial strains (15 species) over 4 hours in the presence of substrates for the detection of alpha and beta galactosidase activity

Organism	No of strains	4-amino-GAL (%positive)	DCAP-GAL (%positive)	ONPG (%positive)	ONPAGAL (%positive)	4-amino-AGAL (%positive)
Escherichia.coli	10	100	100	100	100	100
Klebsiella pneumoniae	10	100	100	100	100	100
Providencia rettgeri	6	0	0	0	0	0
Enterobacter cloacae	10	80	100	100	100	100
Serratia marcescens	10	100	100	100	20	20
Salmonella typhimurium	10	0	0	0	90	90
Pseudomonas aeruginosa	10	0	0	0	0	0
Staphylococcus epidermidis	10	0	0	0	0	0
Streptococcus pyogenes	10	0	0	0	0	0
Enterococcus faecalis	10	0	0	0	0	0
Enterococcus faecium	10	0	0	0	0	0
Listeria monocytogenes	4	100	100	100	0	0
Staphylococcus aureus	10	0	0	0	0	0
Proteus mirabilis	10	0	0	0	0	0
Yersinia enterocolitica	2	100	100	100	100	100

Figure 3.17. Reaction of 15 NCTC strains, *Escherichia.coli* NCTC 10418, *Klebsiella pneumoniae* NCTC 10896, *Providencia rettgeri* NCTC 7475, *Enterobacter cloacae* NCTC 11936, *Serratia marcescens* NCTC 10211, *Salmonella typhimurium* NCTC 74, *Pseudomonas aeruginosa* NCTC 10662, *Staphylococcus epidermidis* NCTC 11047, *Streptococcus pyogenes* NCTC 8306, *Enterococcus faecalis* NCTC 755, *Enterococcus faecium* NCTC 7171, *Listeria monocytogenes* NCTC 11994, *Staphylococcus aureus* NCTC 6571, *Proteus mirabilis* NCTC 10975, *Yersinia enterocolitica* NCTC 11176, and a cell free control well in the presence of 4-aminophenyl-β-D-galactoside (Columns 1+2, wells A-H), 2,6-dichloro-4-aminophenyl-β-D-galactoside (Columns 4+5, wells A-H), and ONPG (Columns 7+8, wells A-H).

Of the β-galactosidase substrates analysed using purified *E.coli* β-galactosidase, it would have been expected from K_m and V_{max} data that the 4-amino-GAL substrate would have performed better than the other 2 substrates. Based on the reciprocal K_{cat} values for this enzyme using 4-amino-GAL (2.8×10^{-4}) which is higher than that observed for ONPG, it would have been expected that 4-amino-GAL would have produced positive reactions both more quickly and more intense in colour than that observed with ONPG. For most test organisms this was indeed the case, the exception being 2 strains of *E.cloacae.*

The calculated K_{cat}/K_m for each substrate showed 4-amino-GAL has the highest efficiency for purified *E.coli* β-galactosidase and might have been expected to produce the best results if extrapolated to the other members of the Enterobacteriaceae. Generally this was observed with the exception of *E.cloacae,* which produced a visibly weaker reaction than that observed in the wells containing ONPG. Indeed a strong positive result was only observed after overnight incubation. Other factors may be equally as important in the overall production of a positive reaction, including differences in substrate uptake and/or mechanisms involving product release/diffusion.

Evaluation of 4-aminophenyl-β-D-xyloside (4-amino-XYL) and *o*-nitrophenyl-β-D-xyloside (ONPXYL) in liquid format.

The results of this experiment are summarised in Table 3.6. This substrate was found to react well with all strains of both *E.cloacae* and *K.pneumoniae.* A red colour was observed after 2 h incubation. Overall for all Gram-negative bacteria tested, the correlation between this substrate and the nitrophenolic derivative was 100%. All strains of *E.faecalis* produced a delayed reaction after overnight incubation. The nitrophenolic derivative *o*-nitrophenyl-β-D-xyloside (ONPXYL) produced a visual positive result around 1 hour faster than with aminophenolic derivatives. This would indicate that the reaction of the aminophenolic substrate was slower, perhaps in small part due to the fact that the released aminophenol has to undergo a subsequent coupling with naphthol before a positive reaction was visible. More likely however is that the delayed reaction may be due to the enzyme β-xylosidase having a higher affinity for the nitrophenyl substrate than the aminophenolic, or difficulties in substrate uptake and/or product release through the cell into the surrounding medium. Based on previous results, if the results of the β-galactosidase enzyme kinetic experiments were extrapolated to β-xylosidase substrates, it would have been expected that the 4-aminophenyl derivative would have produced results faster than the nitrophenolic substrate. The fact that this was not borne out experimentally would suggest the β-galactosidase results couldn't be used

as an indication of how all β-glycosidases behave towards these novel substrates. An avenue of future research would be to examine how each β-glycosidase performs using these novel substrates, in comparison to more commonly used chromogenic equivalents.

Table 3.6. Reaction of 132 bacterial strains (15 species) over 4 hours in the presence of two substrates for the detection of beta xylosidase activity

Organism	No of strains	ONPXYL (% positive)	4-amino-XYL (% positive)
Escherichia.coli	10	0	0
Klebsiella pneumoniae	10	100	100
Providencia rettgeri	6	0	0
Enterobacter cloacae	10	100	100
Serratia marcescens	10	0	0
Salmonella typhimurium	10	0	0
Pseudomonas aeruginosa	10	0	0
Staphylococcus epidermidis	10	0	0
Streptococcus pyogenes	10	0	0
Enterococcus faecalis	10	100*	100
Enterococcus faecium	10	0	0
Listeria monocytogenes	4	0	0
Staphylococcus aureus	10	0	0
Proteus mirabilis	10	0	0
Yersinia enterocolitica	2	0	0

* Required overnight incubation before positive reaction was observed.

Evaluation of 4-aminophenyl-β-D-glucuronide (4-amino-GUR), 4-amino-2,6-dichlorophenyl-β-D-glucuronide (DCAP-GUR), and *o*-nitrophenyl-β-D-glucuronide (ONPGUR) in liquid format.

Initially the first batch of the halogenated derivative was not hydrolysed by any of the 132 strains tested. This was most likely to be a synthetic problem involving the inadequate removal of a methyl group from the carboxyl group of the glucuronic acid residue (James, personal communication). The substrate was re-synthesised and examined under the same experimental conditions. The results of this experiment are summarised in Table 3.7. Unsubstituted 4-amino-GUR produced deep red coloration with 80% strains of *E.coli* in 2-3 hours, and a range of other Gram-negative bacteria as expected, produced no reaction. The correlation with the nitrophenolic derivative was absolute, indicating the strains of *E.coli,* negative with the aminophenolic substrate failed to produce β-glucuronidase. A negative β-glucuronidase reaction occurs in *E.coli* strains belonging to the serogroup O157 (Monday *et al.,* 2001), a negative reaction was however recorded with *E.coli* O157 antisera for the β-glucuronidase negative strains. Presumably these are β-glucuronidase negative *E.coli* belonging to a serogroup other than O157. The halogenated derivative produced a blue colour in the positive wells. The results of this substrate were in complete agreement with those obtained for both the unsubstituted derivative and the nitrophenolic substrate. Interestingly, whilst the nitrophenolic derivative

generally produced visible positive results faster than either aminophenolic derivative this was not universal. Indeed 2 of the strains of *E.coli,* positive for β-glucuronidase activity, produced a positive result in the wells containing the halogenated aminophenolic substrate first. This was evident after 1 hour for the aminophenolic substrate, with the nitrophenolic substrate producing a visible reaction after 2-3 hours incubation. Since this cannot be explained by any possible differences in innoculum, it is likely that dissimilar enzyme efficiencies and/or uptake differences have produced these variations in reaction results. Further research would be required to explain these discrepancies. Overall both aminophenol substrates appear to be promising for the detection of bacterial ß-glucuronidase activity (Table 3.7). The fact that these novel substrates were hydrolysed before the corresponding nitrophenolic substrate, as judged visibly, indicates a potential rapid diagnostic use.

Table 3.7. Reaction of 132 bacterial strains (15 species) in the presence of three substrates for the detection of beta-glucuronidase activity

Organism	No of strains	4-amino-GUR (% positive)	DCAP-GUR (% positive)	ONPGUR (% positive)
Escherichia.coli	10	80	80	80
Klebsiella pneumoniae	10	0	0	0
Providencia rettgeri	6	0	0	0
Enterobacter cloacae	10	0	0	0
Serratia marcescens	10	0	0	0
Salmonella typhimurium	10	0	0	0
Pseudomonas aeruginosa	10	0	0	0
Staphylococcus epidermidis	10	0	0	0
Streptococcus pyogenes	10	0	0	0
Enterococcus faecalis	10	0	0	0
Enterococcus faecium	10	0	0	0
Listeria monocytogenes	4	0	0	0
Staphylococcus aureus	10	0	0	0
Proteus mirabilis	10	0	0	0
Yersinia enterocolitica	2	0	0	0

Evaluation of 4-aminophenyl-β-D-glucoside (4-amino-GLU), 4-amino-2,6-dichlorophenyl-β-D-glucoside (DCAP-GLU), and *o*-nitrophenyl-β-D-glucoside (ONPGLU) in liquid format.

Initial experiments showed the un-halogenated substrate was hydrolysed in less than an hour by a range of bacteria to produce a yellow coloration. Production of the yellow colour correlated exactly with the production of ß-glucosidase in control wells using *o*-nitrophenyl-ß-D-glucoside (ONPGLU). The production of the yellow coloration from hydrolysis of this compound indicated a synthetic problem in which the starting material (ONPGLU) had not been successfully reduced to form the 4-aminophenyl derivative. Since this became a recurring problem, a commercial sample of 4-amino-GLU (Sigma) was purchased and the experiments repeated. The results of this experiment are shown in Table 3.8 and Figure 3.18.

Table 3.8. Reaction of 132 bacterial strains (15 species) over 4 hours in the presence of three substrates for the detection of beta-glucosidase activity

Organism	No of strains	4-amino-GLU (% positive)	DCAP-GLU (% positive)	ONPGLU (% positive)
Escherichia.coli	10	0	10*	0
Klebsiella pneumoniae	10	0	100	100
Providencia rettgeri	6	0	0	0
Enterobacter cloacae	10	0	90	90
Serratia marcescens	10	0	90	90
Salmonella typhimurium	10	0	10*	0
Pseudomonas aeruginosa	10	0	0	0
Staphylococcus epidermidis	10	0	0	0
Streptococcus pyogenes	10	0	0	0
Enterococcus faecalis	10	100	100	100
Enterococcus faecium	10	100	100	100
Listeria monocytogenes	4	100	100	100
Staphylococcus aureus	10	0	0	0
Proteus mirabilis	10	0	0	0
Yersinia enterocolitica	2	0	0	0

** Required overnight incubation before positive reaction was observed.*

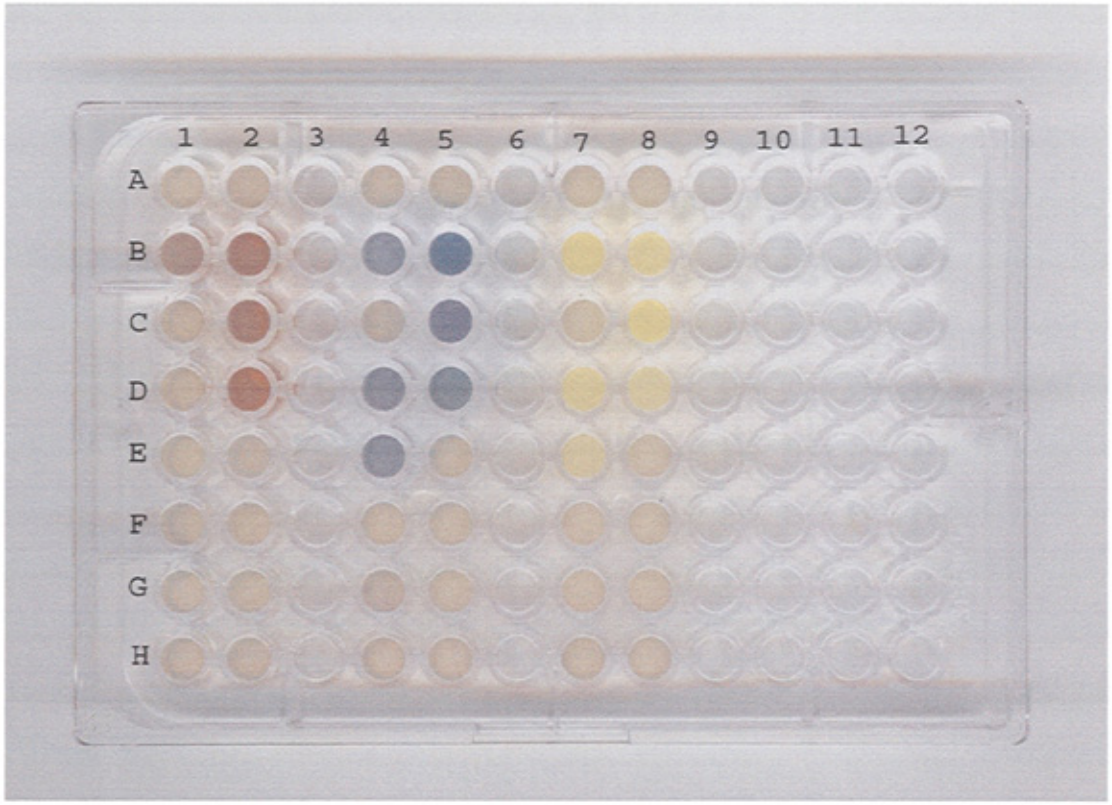


Figure 3.18. Reaction of 15 NCTC strains, *Escherichia.coli* NCTC 10418, *Klebsiella pneumoniae* NCTC 10896, *Providencia rettgeri* NCTC 7475, *Enterobacter cloacae* NCTC 11936, *Serratia marcescens* NCTC 10211, *Salmonella typhimurium* NCTC 74, *Pseudomonas aeruginosa* NCTC 10662, *Staphylococcus epidermidis* NCTC 11047, *Streptococcus pyogenes* NCTC 8306, *Enterococcus faecalis* NCTC 755, *Enterococcus faecium* NCTC 7171, *Listeria monocytogenes* NCTC 11994, *Staphylococcus aureus* NCTC 6571, *Proteus mirabilis* NCTC 10975, *Yersinia enterocolitica* NCTC 11176, and a cell free control well in the presence of 4-aminophenyl-β-D-glucoside (Columns 1+2, wells A-H), 4-amino-2,6-dichlorophenyl-β-D-glucoside (Columns 4+5, wells A-H), and *o*-nitrophenyl-β-D-glucoside (Columns 7+8, wells A-H).

Using 4-amino-GLU, although a red/orange colour was generated by some organisms, notably *Enterococcus* spp. and *Listeria* spp. (B:2-D:2). Other known ß-glucosidase producing strains such as *S.marcescens* (E:1) were negative and *K.pneumoniae* produced only a very weak reaction (B:1). The di-chloro derivative was hydrolysed by ß-glucosidase producing strains forming a blue coloration in the test wells. The best results were recorded with Gram-positive organisms such as *L.monocytogenes*, *E.faecalis* and *E.faecium* (B:5-D:5), which all produced a blue coloration within the first hour of incubation. Over 4 hours incubation, correlation with ONPGLU was absolute. One unusual finding was that one strain of *E.coli* (NCTC 10418) and one wild strain of *Salmonella* sp. produced a positive reaction after overnight incubation but remained negative using the nitrophenolic derivative (Table 3.8). Overall the unsubstituted 4-aminophenol substrate is of little use for the detection of β-glucosidase activity in Gram-negative organisms, but excellent for use with Gram-positive strains. Since these Gram-negative strains produced a positive reaction in the wells containing the nitrophenolic derivative, it would again suggest difficulties in either uptake or in substrate hydrolysis, resulting in a failure of the compound to gain entry into or exit from the cell. This is reinforced from the fact the dichloro substrate works as well as the conventional chromogenic substrate. Based on these results it is possible that the presence of 2 chlorine atoms may facilitate a faster entry into the cell. It is also possible, however, that both compounds are transported into the cell and that upon cleavage the

presence of two chlorine atoms facilitates diffusion back across the cytoplasmic membrane.

Evaluation of 4-aminophenyl-α-D-fucoside (4-amino-AFUC), 4-aminophenyl-β -D-fucoside (4-amino-BFUC), *o*-nitrophenyl-α-D-fucoside (ONPAFUC), and *o*-nitrophenyl-β-D-fucoside (ONPBFUC).

The full results are shown in Table 3.9. This experiment, for α-D-fucosidase activity showed that none of the 132 test strains produced positive results with either the aminophenolic and nitrophenolic substrates, even on prolonged incubation. This experiment indicates that α-D-fucosidase activity is absent in Enterobacteriaceae and in several Gram-positive species. Organisms, which possess this activity, include *Bacteroides* spp (Dellinger and Moore, 1986). Therefore the tests were repeated with four *Bacteroides* ACTC strains. Of these only *Bacteroides vulgatus* (ACTC 8482) produced a positive reaction with a typical red coloration after 3 hours incubation, and a positive reaction with the nitrophenolic derivative as judged by the release of yellow *o*-nitrophenol. This indicates that 4-aminophenyl-α-D-fucoside may have some use as a substrate for the differentiation amongst the genus Bacteroides, although more strains of *Bacteroides* sp need to be analysed before the potential use of this substrate can be evaluated.

The substrate 4-aminophenyl-β-D-fucoside showed a limited reactivity in initial screening with only strains of *K.pneumoniae*, and *E.faecium* producing a positive reaction with both aminophenolic and nitrophenolic

substrates. The range of organisms tested also included the four *Bacteroides* strains, of these only *Bacteroides vulgatus* (ACTC 8482) hydrolysed the substrate to produce a red colouration after 4 hours. *Bacteroides distasonis* (ATCC 8503), however, produced a negative reaction with this substrate but was shown to rapidly hydrolyse *o*-nitrophenyl-ß-D-fucoside. This substrate shows potential for the differentation of *E.faecalis* from *E.faecium* and *K.pneumoniae* from other members of the enterobacteriaceae. Due to the limited reactivity of these substrates photographs were not produced.

Table 3.9. Reaction of 136 bacterial strains (16 species) over 4 hours in the presence substrates for the detection of alpha and beta-fucosidase activity

Organism	No of strains	4-amino-AFUC (% positive)	ONPAFUC (% positive)	4-amino-BFUC (% positive)	ONPBFUC (% positive)
Escherichia coli	10	0	0	0	0
Klebsiella pneumoniae	10	0	0	100	100
Providencia rettgeri	6	0	0	0	0
Enterobacter cloacae	10	0	0	0	0
Serratia marcescens	10	0	0	0	0
Salmonella typhimurium	10	0	0	0	0
Pseudomonas aeruginosa	10	0	0	0	0
Staphylococcus epidermidis	10	0	0	0	0
Streptococcus pyogenes	10	0	0	0	0
Enterococcus faecalis	10	0	0	0	0
Enterococcus faecium	10	0	0	100	100
Listeria monocytogenes	4	0	0	0	0
Staphylococcus aureus	10	0	0	0	0
Proteus mirabilis	10	0	0	0	0
Yersinia enterocolitica	2	0	0	0	0
Bacteroides vulgatis (ACTC 8482)	1	100	100	100	100
Bacteroides distasonis (ATCC 8503)	1	0	0	0	100
Bacteroides fragilis (ATCC 25285)	1	0	0	0	0
Bacteroides ovatus (ATCC 8483)	1	0	0	0	0

Evaluation of 4-aminophenyl-α-D-glucoside (4-amino-AGLU), and 4-aminophenyl-N-acetyl-β-D-glucosamine (4-amino-NAG), *p*-nitrophenyl-α-D-glucoside (PNPAGLU), and *p*-nitrophenyl-N-acetyl-β-D-glucosaminide (PNPNAG).

Table 3.10 shows the complete results of this experiment. The substrate 4-amino-AGLU was rapidly hydrolysed by a diverse range of strains and the pattern was matched almost perfectly when compared with *p*-nitrophenyl-α-D-glucoside. A positive reaction observed with all *Listeria, Salmonella,* and *Klebsiella* sp tested. In addition 90% of test *E.cloacae,* and *S.marcescens* strains were positive for α-glucosidase activity. The only exception to this was one strain of *E.coli,* which hydrolyses the *p*-nitrophenyl derivative and not the 4-aminophenyl derivative. This finding is interesting since *E.coli* are not known to be producers of α-glucosidase.

The N-acetyl derivative failed to give a visible reaction with any of the bacterial strains tested. Because of the potential value of this substrate for the differentiation of *Candida* spp the experiment was repeated with a range of *Candida* NCPF and ATCC strains. A weak positive reaction was produced by two strains of *C.albicans,* and one strain of *C.tropicalis* but only following overnight incubation using the 4-aminophenol substrate. No coloured reaction product was observed after 4 hours incubation. Positive reactions with the nitrophenyl derivative were observed with only one strain of *C.albicans. C.albicans* ACTC 90028 and *C.tropicalis* NCPF 3980,

which produced a positive reaction with the aminophenol substrate, failed to produce a positive reaction in the wells containing the nitrophenolic. Overall this substrate would appear to be limited in its ability to differentiate members of the *Candida* genus, and as such further work with these substrates was not continued.

Table 3.10. Reaction of 144 bacterial strains (16 species) over 4 hours for the detection of alpha glucosidase and N-acetyl-beta-galactosidase activity

Organism	No of strains	4-amino-AGLU (% positive)	PNPAGLU (% positive)	4-amino-NAG (% positive)	PNPNAG (% positive)
Escherichia.coli	10	0	10	0	0
Klebsiella pneumoniae	10	100	100	0	0
Providencia rettgeri	6	0	0	0	0
Enterobacter cloacae	10	90	90	0	0
Serratia marcescens	10	90	90	0	0
Salmonella typhimurium	10	100	100	0	0
Pseudomonas aeruginosa	10	0	0	0	0
Staphylococcus epidermidis	10	0	0	0	0
Streptococcus pyogenes	10	0	0	0	0
Enterococcus faecalis	10	0	0	0	0
Enterococcus faecium	10	0	0	0	0
Listeria monocytogenes	4	100	100	0	0
Staphylococcus aureus	10	0	0	0	0
Proteus mirabilis	10	0	0	0	0
Yersinia enterocolitica	2	100	100	0	0
Candida albicans (ATCC 90028)	1	NT	NT	100*	100
Candida albicans (ATCC 90029)	1	NT	NT	0	0
Candida albicans (ATCC 64547)	1	NT	NT	0	0
Candida albicans (ATCC 64551)	1	NT	NT	100*	0
Candida albicans (ATCC 64553)	1	NT	NT	0	0
Candida albicans (NCPF 3281)	1	NT	NT	0	0
Candida glabrata (NCPF 3943)	1	NT	NT	0	0
Candida glabrata (NCPF 8018)	1	NT	NT	0	0
Candida krusei (NCPF 3953)	1	NT	NT	0	0
Candida lusitaniae (NCPF 3945)	1	NT	NT	0	0
Candida parapsilosis (NCPF 3938)	1	NT	NT	0	0
Candida tropicalis (NCPF 3980)	1	NT	NT	100	0

** Required overnight incubation before positive reaction was observed.*

Aminopeptidase substrates

Evaluation of L-alanyl-4-aminophenol (L-ala-4-amino), L-alanyl-4-amino-2,6-dichlorophenol (L-ala-DCAP), L-alanyl-DEPPD (L-ala-DEPPD), and L-alanyl-*p*-nitroanilide (L-ala-*p*NA) in liquid format.

The full results of this experiment are shown in Table 3.11, and illustrated in Figure 3.19. All of the test substrates were all rapidly hydrolysed by all Gram-negative bacteria tested to produce strong colours with 3,5-dihydroxy-2-naphthoic acid. As seen from Figure 3.19, all strains of *E.coli, K.pneumoniae, P.rettgeri, E.cloacae, S.marcescens, S.typhimurium, P.aeruginosa, P.mirabilis* and *Y.enterocolitica* produced strong positive results with all of the test substrates. Of the Gram-positive isolates tested only *S.epidermidis* and *S.aureus* produced a negative reaction with all substrates. Some wild strains of *E.faecium, E.faecalis,* and *L.monocytogenes* produced a positive reaction with all of the substrates examined, and the strains producing the positive results were the same for all of the test substrates. These reactions are surprising since *E.faecium, E.faecalis,and L.monocytogenes* are not known to be producers of L-alanyl-aminopeptidase. The reaction of *L.monocytogenes* was visibly stronger with all of the aminophenol substrates, compared to the nitrophenolic derivative. In addition the NCTC control strain of *S.pyogenes* produced a very strong reaction with all four substrates. It is again interesting as this species is not known to produce L-alanyl aminopeptidase, and that the strain used to

typify the genus is biochemically atypical in this respect. Overall weak reactions were easier to visualise with all of the novel test substrates, than with the nitrophenolic derivative. The results were in complete agreement with those observed with L-alanyl-*p*-NA. Although very weak reactions were observed with the test substrates and some other Gram-positive strains, these were not evident in the wells containing the corresponding *p*-nitroanilide substrate. From these results all three novel substrates appear to be useful for the detection of L-alanyl-aminopeptidase (Table 3.11).

Table 3.11. Reaction of 132 bacterial strains (15 species) in the presence of 5 substrates for the detection of alanyl aminopeptidase activity

Organism	No of strains	L-ala-DCAP (% positive)	L-ala-4-amino (% positive)	L-ala-DEPPD (% positive)	L-ala-*p*-NA (% positive)
Escherichia.coli	10	100	100	100	100
Klebsiella pneumoniae	10	100	100	100	100
Providencia rettgeri	6	100	100	100	100
Enterobacter cloacae	10	100	100	100	100
Serratia marcescens	10	100	100	100	100
Salmonella typhimurium	10	100	100	100	100
Pseudomonas aeruginosa	10	100	100	100	100
Staphylococcus epidermidis	10	0	0	0	0
Streptococcus pyogenes	10	100	100	100	100
Enterococcus faecalis	10	100	100	100	0
Enterococcus faecium	10	100	100	100	100
Listeria monocytogenes	4	100	100	100	0
Staphylococcus aureus	10	0	0	0	0
Proteus mirabilis	10	100	100	100	100
Yersinia enterocolitica	2	100	100	100	100

Figure 3.19. Reaction of 15 NCTC strains, *Escherichia.coli* NCTC 10418, *Klebsiella pneumoniae* NCTC 10896, *Providencia rettgeri* NCTC 7475, *Enterobacter cloacae* NCTC 11936, *Serratia marcescens* NCTC 10211, *Salmonella typhimurium* NCTC 74, *Pseudomonas aeruginosa* NCTC 10662, *Staphylococcus epidermidis* NCTC 11047, *Streptococcus pyogenes* NCTC 8306, *Enterococcus faecalis* NCTC 755, *Enterococcus faecium* NCTC 7171, *Listeria monocytogenes* NCTC 11994, *Staphylococcus aureus* NCTC 6571, *Proteus mirabilis* NCTC 10975, *Yersinia enterocolitica* NCTC 11176, and a cell free control well in the presence of L-ala-DEPPD (Column 1+2), L-ala-4-aminophenol (Column 4+5), L-ala-DCAP (Column 7+8), and L-ala-*p*-NA (Column 10+11).

Evaluation of L-pyroglutamyl-4-amino-2,6-dichlorophenol (L-pyra-DCAP) and L-pyroglutamyl-*p*-nitroanilide (L-pyra-*p*NA).

The results of this experiment are shown in Table 3.12. All strains of *E.faecalis* and *E.faecium* and *S.pyogenes* produced positive results in one hour. In addition all *K.pneumoniae* and *S.aureus* strains produced a positive result for L-pyroglutamyl aminopeptidase. Of the Gram-negative bacteria tested, 3 strains of *E.cloacae* produced a weak reaction after overnight incubation. This was observed only with the DCAP substrate. The results were in complete agreement with those obtained with L-pyroglutamic acid-*p*-nitroanilide. From these results L-pyroglutamyl-4-amino-2,6-dichlorophenol would appear to be an excellent substrate for the detection of L-pyroglutamyl aminopeptidase.

Evaluation of β-Alanyl-4-amino-2,6-dichlorophenol (β-DCAP).

The full results of this experiment are shown in Table 3.13. Since no corresponding *p*-nitroanilide substrate is available for the detection of β-alanyl aminopeptidase it was decided to investigate the reactivity of this substrate to determine if the substrate was useful in the differentiation of non-Enterobacteriaceae in addition to the 132 strains used in the assay of other substrates. Using the additional range of non-Enterobacteriaceae strains, positive reactions were observed after 2 hours incubation in wells containing *P.aeruginosa* (10 strains), *P.putida* (1 strain), *M.nonliquefaciens*

(1 strain) and *B.cepacia* (1 strain). After the 3 hours of incubation *M.osloensis (1 strain)* and *P.fluorescens* (1 strain) also produced a positive reaction. No further positive reactions were observed after overnight incubation. All strains of Enterobacteriaceae and Gram-positive control strains failed to produce a positive reaction. Despite the lack of a *p*-nitroanilide control substrate for comparison, β-alanyl-4-amino-2,6-dichlorophenol appears to be a promising stable substrate for the detection of bacterial β-alanyl aminopeptidase activity, and the differentiation of organisms considered difficult to separate by conventional identification systems.

Table 3.12. Reaction of 132 bacterial strains (15 species) over four hours in the presence of 2 substrates for the detection of L-pyroglutamyl aminopeptidase activity

Organism	No of strains	L-pyra-DCAP (% positive)	L-pyra-*p*-NA (% positive)
Escherichia.coli	10	0	0
Klebsiella pneumoniae	10	0	0
Providencia rettgeri	6	0	0
Enterobacter cloacae	10	30	0
Serratia marcescens	10	0	0
Salmonella typhimurium	10	0	0
Pseudomonas aeruginosa	10	0	0
Staphylococcus epidermidis	10	0	0
Streptococcus pyogenes	10	100	100
Enterococcus faecalis	10	100	100
Enterococcus faecium	10	100	100
Listeria monocytogenes	4	0	0
Staphylococcus aureus	10	0	0
Proteus mirabilis	10	0	0
Yersinia enterocolitica	2	0	0

Table 3.13. Reaction of 148 bacterial strains (21 species) over 4 hours in the presence of beta-alanyl-4-amino-2,6-dichlorophenol

Organism	No of strains (% positive)	beta-ALA-DCAP (% positive)
Escherichia coli	10	0
Klebsiella pneumoniae	10	0
Providencia rettgeri	6	0
Enterobacter cloacae	10	0
Serratia marcescens	10	0
Salmonella typhimurium	10	0
Pseudomonas aeruginosa	10	100
Staphylococcus epidermidis	10	0
Streptococcus pyogenes	10	0
Enterococcus faecalis	10	0
Enterococcus faecium	10	0
Listeria monocytogenes	4	0
Staphylococcus aureus	10	0
Proteus mirabilis	10	0
Yersinia enterocolitica	2	0
Pseudomonas stutzeri ATCC 17588T	1	0
Burkholderia cepacia ATCC 27511T	1	100
Brevundimonas vesicularis ATCC 114426T	1	0
Pseudomonas fluorescens ATCC 13525	1	100
Pseudomonas alcaligenes ATCC 14909T	1	0
Shewanella putrefaciens ATCC 8071T	1	0
Pseudomonas putida ATCC 12633T	1	0
Moraxella osloensis ATCC 19976T	1	100
Acinetobacter haemolyticus ATCC 17906T	1	0
Acinetobacter baumannii ATCC 19606T	1	0
Acinetobacter johnsonii ATCC 17909T	1	0
Acinetobacter lwoffii ATCC 15309T	1	0
Brevundimonas diminuta ATCC 11568T	1	0
Moraxella nonliquefaciens ATCC 19975T	1	100
Pseudomonas aeruginosa ATCC 10145	1	100
Burkholderia cepacia ATCC 25416T	1	100

Evaluation of Leucyl-4-amino-2,6-dichlorophenol (L-Leu-DCAP), and L-leucine-*p*-nitroanilide (L-leu-*p*-NA)

All of the Gram-negative strains tested produced a rapid positive reaction with this substrate (Figure 3.20). Of the Gram-positive organisms, only *S.pyogenes* was strongly reactive, producing a strong blue coloration after 1 hours incubation (A:2). All of the reactive strains produced colour within 4 hours and this intensified after overnight incubation. It was surprising to note that both species of *Enterococcus* (B2:C2) produced only very weak reactions with the halogenated substrate in four hours, as enterococci are known to be strong producers of leucyl aminopeptidase (Facklam *et al.*, 1995). Results obtained with L-leu-*p*-NA were positive for all of the Gram-negative strains tested, and strong results as expected for all enterococci and *S.pyogenes* strains. The target organisms for this substrate are streptococci, in particular *S.pyogenes* and enterococci. The full results of this experiment are shown in Table 3.14. The fact that the novel substrate produced weak reactions for these species suggests a limited used for the substrate in liquid media.

Table 3.14. Reaction of 132 bacterial strains (15 species) over 4 hours in the presence of 2 substrates for the detection leucyl aminopeptidase activity

Organism	No of strains	L-leu-DCAP (% positive)	L-leu-*p*-NA (% positive)
Escherichia coli	10	100	100
Klebsiella pneumoniae	10	100	100
Providencia rettgeri	6	100	100
Enterobacter cloacae	10	100	100
Serratia marcescens	10	100	100
Salmonella typhimurium	10	100	100
Pseudomonas aeruginosa	10	100	100
Staphylococcus epidermidis	10	0	0
Streptococcus pyogenes	10	100	100
Enterococcus faecalis	10	0	100
Enterococcus faecium	10	0	100
Listeria monocytogenes	4	0	0
Staphylococcus aureus	10	0	0
Proteus mirabilis	10	100	100
Yersinia enterocolitica	2	100	100

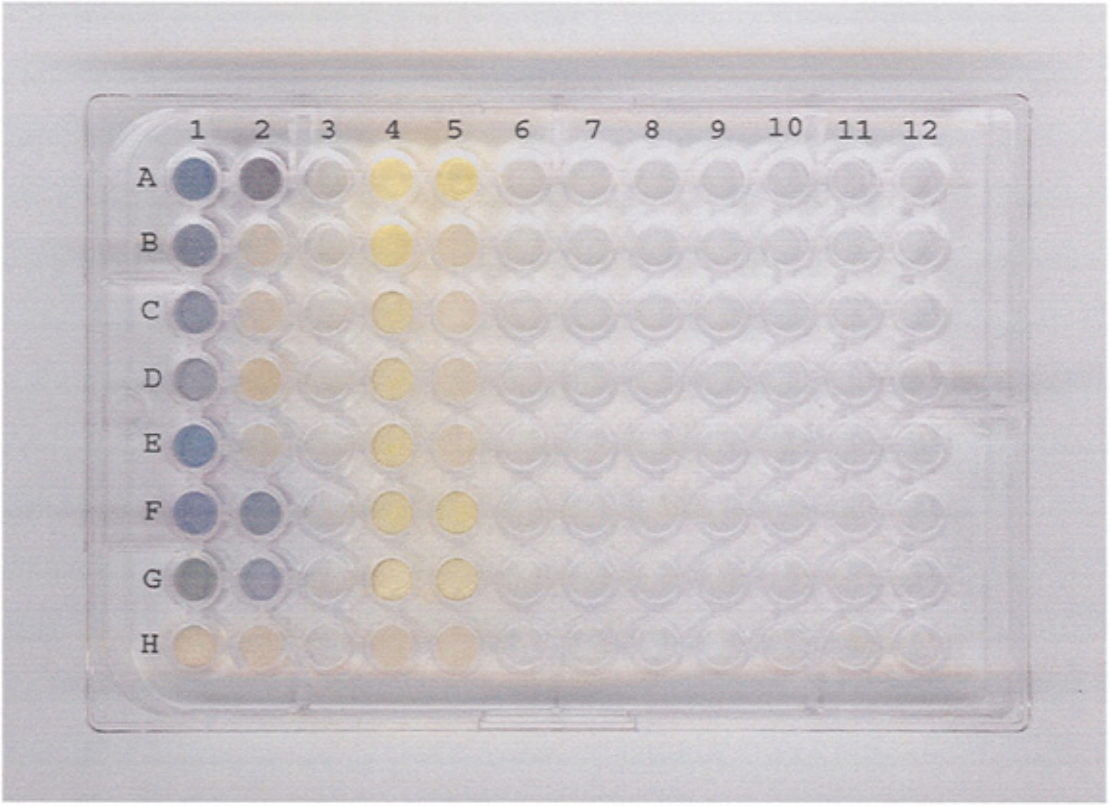


Figure 3.20. Reaction of 15 NCTC strains, *Escherichia.coli* NCTC 10418, *Klebsiella pneumoniae* NCTC 10896, *Providencia rettgeri* NCTC 7475, *Enterobacter cloacae* NCTC 11936, *Serratia marcescens* NCTC 10211, *Salmonella typhimurium* NCTC 74, *Pseudomonas aeruginosa* NCTC 10662, *Staphylococcus epidermidis* NCTC 11047, *Streptococcus pyogenes* NCTC 8306, *Enterococcus faecalis* NCTC 755, *Enterococcus faecium* NCTC 7171, *Listeria monocytogenes* NCTC 11994, *Staphylococcus aureus* NCTC 6571, *Proteus mirabilis* NCTC 10975, *Yersinia enterocolitica* NCTC 11176, and a cell free control well in the presence of L-leucyl-4-amino-2,6-dichlorophenol (Column 1+2, wells A-H), and L-leucyl-*p*-nitroanilide (Column 4+5, wells A-H).

Phosphatase substrates

Evaluation of 4-aminophenyl-phosphate (4-APP), 4-aminophenyl-phosphate-tetra ethyl ammonium salt (4-APP-TEA), 4-amino-2,6-dichlorophenyl phosphate (DCAP-Phos), and *o*-nitrophenyl phosphate (ONP-Phos) in liquid format.

The results of this experiment are shown in Table 3.15. Only strains of *K.pneumoniae* and *E.cloacae* produced positive reactions in the wells containing 4-aminophenyl-phosphate after four hours incubation. No strain of *S.aureus* hydrolysed this compound after four hours. The TEA salt of the same substrate produced a dark-brown solution upon dissolving which interfered with the reading of the orange colour produced when hydrolysed. Only *K.pneumoniae* strains hydrolysed this substrate in four hours. Due to the poor correlation between the *p*-nitrophenol derivative and the difficulties associated in differentiating positive reactions from background, this substrate has little use for detection of phosphatase activity. The di-chloro derivative was difficult to dissolve and required gentle heating. The effect of heating changed the solution from light green to dark brown. All organisms, including the un-inoculated control, rapidly produced a blue/black colour indicating the unstable nature of the compound due to the presence of free 4-amino-2,6-dichlorophenol. Using ONP-Phos all test bacteria with the exception of *S.pyogenes*, *E.facealis*, and *E.faecium*, demonstrated phosphatase activity. These results indicated that none of the

novel substrates are useful for the detection of bacterial phosphatase activity. The results of these substrates are shown in table 3.15.

Table 3.15. Reaction of 132 bacterial strains (15 species) over 4 hours in the presence of 4 substrates for the detection of phosphatase activity

Organism	No of strains	4-APP (% positive)	4-APP-TEA (% positive)	DCAP-Phos (% positive)	ONP-Phos (% positive)
Escherichia coli	10	0	0	0	100
Klebsiella pneumoniae	10	100	100	0	100
Providencia rettgeri	6	0	0	0	100
Enterobacter cloacae	10	100	100	0	100
Serratia marcescens	10	0	0	0	100
Salmonella typhimurium	10	0	0	0	100
Pseudomonas aeruginosa	10	0	0	0	100
Staphylococcus epidermidis	10	0	0	0	100
Streptococcus pyogenes	10	0	0	0	0
Enterococcus faecalis	10	0	0	0	0
Enterococcus faecium	10	0	0	0	0
Listeria monocytogenes	4	0	0	0	100
Staphylococcus aureus	10	0	0	0	100
Proteus mirabilis	10	0	0	0	100
Yersinia enterocolitica	2	0	0	0	100

Miscellaneous substrates

Evaluation of 4-aminophenyl-phosphorylcholine (4-amino-PC) and *p*-nitrophenyl-phosphorylcholine (PNPPC)

This compound, 4-aminophenyl-phosphorylcholine (Figure 3.21) produced no visible reaction with any of the NCTC strains initially tested, even after overnight incubation.

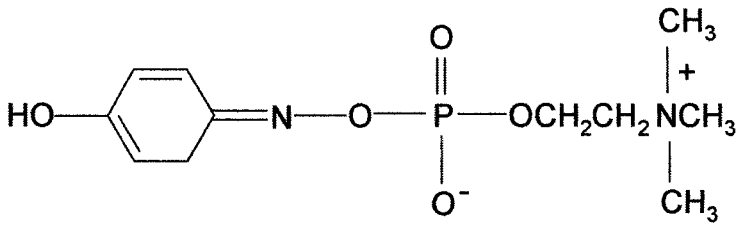


Figure 3.21. Chemical structure of 4-aminophenyl-phosphorylcholine

This was in spite of the fact that the *o*-nitrophenyl derivative was rapidly hydrolysed by six species of the Enterobacteriaceae (Table 3.16). These included strains of *E.coli*, *K.pneumoniae*, *E.cloacae*, *S.marcescens*, and *Salmonella* sp. No single species produced 100% positive results, indicating phosphorylcholine esterase is not universal among members of the Enterobacteriaceae. Only *L.monocytogenes* of the Gram-positive species tested produced a positive reaction. Since more appropriate target organisms of this substrate are *Aeromonas* spp, *Bacillus cereus*, and *Haemophilus* spp,

the experiment was repeated with these organisms (Reis *et al.*, 1992). The results of both sets of experiments are summarised in table 3.16. Of the additional strains, only one strain of *Aeromonas* (*A.hydrophila* NCTC 8049) was positive with the nitrophenolic derivative. The strain of *B.cereus* and all five *Haemophilus* strains failed to produce positive results with either substrate. This would indicate that 4-aminophenyl-phosphorylcholine is not hydrolysed by phosphorylcholine esterase and as such is of no use as a substrate for the detection of bacterial strains known to produce this enzyme. Because of the poor reactivity of this substrate no photographs were produced and no further work was carried out.

Table 3.16. Reaction of 142 bacterial strains (18 species) over 4 hours in the presence of 2 substrates for the detection of phosphorylcholine esterase

Organism	No of strains	4-amino-PC (% positive)	PNPPC (% positive)
Escherichia.coli	10	0	70
Klebsiella pneumoniae	10	0	50
Providencia rettgeri	6	0	0
Enterobacter cloacae	10	0	80
Serratia marcescens	10	0	60
Salmonella typhimurium	10	0	60
Pseudomonas aeruginosa	10	0	0
Staphylococcus epidermidis	10	0	0
Streptococcus pyogenes	10	0	0
Enterococcus faecalis	10	0	0
Enterococcus faecium	10	0	0
Listeria monocytogenes	4	0	100
Staphylococcus aureus	10	0	0
Proteus mirabilis	10	0	0
Yersinia enterocolitica	2	0	100
Bacillus cereus NCTC 7464	1	0	0
Haemophilus influenzae NCTC 8143	1	0	0
Haemophilus influenzae NCTC 11931	1	0	0
Haemophilus influenzae NCTC 8467	1	0	0
Haemophilus influenzae NCTC 11315	1	0	0
Haemophilus parainfluenzae NCTC 7857	1	0	0
Aeromonas caviae NCTC 10852	1	0	0
Aeromonas hydrophila NCTC 8049	1	0	100
Aeromonas hydrophila NCTC 3249	1	0	0
Aeromonas sobria NCTC 11215	1	0	0

Evaluation of 4-acetamidophenol (4-ACP; Paracetamol), 2,6-dichloro-4-acetamidophenol (DCAP-4-ACP), and *o*-nitrophenyl acetate (ONPA)

This substrate was derivitised to determine if the acetyl group of either of these compounds could be removed by the test organisms. Upon hydrolysis by an acetase enzyme, the acetyl group would be removed to release *p*-aminophenol and acetic acid. The aminophenol would then couple with 3,5-dihydroxy-2-naphthoic acid to produce a red complex or a blue violet complex if the released product was 4-amino-2,6-dichlorophenol.

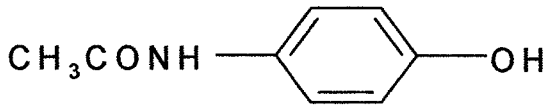


Figure 3.22. Chemical structure of 4-acetamidophenol (Paracetamol).

The results of this experiment are shown in Table 3.17. Three of the 15 NCTC bacteria tested showed weak reactivity with 4-ACP, to produce a faint red coloration over 4 hours incubation. These strains were 4 strains of *S.epidermidis* and 2 strains each of *E.faecalis* and *E.faecium.* In addition 12 *Candida* NCPF and ATCC strains were also examined and two *C.albicans* strains produced weakly positive results. The dichloro derivative appeared to be less readily converted, since none of the bacterial isolates produced a positive reaction, and only one strain of *C.albicans* produced a weak blue reaction product after overnight incubation. The conventional nitrophenolic substrate, ONPA produced positive reactions with all strains

of *E.faecalis*, *E.faecium* and *S.epidermidus*. In addition some strains of *S.marcescens* and 10% of *P.rettgeri* strains produced positive reactions after 4 hours incubation. Of the *Candida* strains, a positive reaction was observed with all strains of *C.albicans* and *C.krusei*. Overall, the derivitisation of paracetamol has failed to produce substrates which are useful for the detection of acetase enzymes. As such further research in this area was not pursued.

Table 3.17. Reaction of 144 bacterial strains (16 species) over 4 hours in the presence of substrates for the detection of acetase activity

Organism	No of strains	4-ACP (% positive)	DCAP-4-ACP (% positive)	ONPA (% positive)
Escherichia.coli	10	0	0	0
Klebsiella pneumoniae	10	0	0	0
Providencia rettgeri	6	0	0	10
Enterobacter cloacae	10	0	0	0
Serratia marcescens	10	0	0	40
Salmonella typhimurium	10	0	0	0
Pseudomonas aeruginosa	10	0	0	0
Staphylococcus epidermidis	10	40	0	100
Streptococcus pyogenes	10	0	0	0
Enterococcus faecalis	10	20	0	100
Enterococcus faecium	10	20	0	100
Listeria monocytogenes	4	0	0	0
Staphylococcus aureus	10	0	0	0
Proteus mirabilis	10	0	0	0
Yersinia enterocolitica	2	0	0	0
Candida albicans (ATCC 90028)	1	0	0	100
Candida albicans (ATCC 90029)	1	100	0	100
Candida albicans (ATCC 64547)	1	0	0	100
Candida albicans (ATCC 64551)	1	0	0	100
Candida albicans (ATCC 64553)	1	100	100*	100
Candida albicans (NCPF 3281)	1	0	0	100
Candida glabrata (NCPF 3943)	1	0	0	0
Candida glabrata (NCPF 8018)	1	0	0	0
Candida krusei (NCPF 3953)	1	0	0	100
Candida lusitaniae (NCPF 3945)	1	0	0	0
Candida parapsilosis (NCPF 3938)	1	0	0	0
Candida tropicalis (NCPF 3980)	1	0	0	0

Evaluation of t-boc-val-pro-arg-DMPPD in comparison with t-boc-val-pro-arg-7-AMC for the detection of staphylocoagulase.

Table 3.18 shows the results of the experiment using the chromogenic test substrate. It is evident that the optimal substrate concentration for detection of staphylocoagulase was 1.5 mg/ml (final concentration) which produced a blue coloured reaction product with all coagulase-positive *S.aureus* strains tested. The test *S.epidemidis* strain was also negative, and as expected no colour was observed in the well containing *E.faecalis*. Table 3.19 shows the results obtained using the fluorescent substrate t-boc-val-pro-arg-7-amido-4-methylcoumarin. These results were in complete agreement with those obtained with the *p*-phenylenediamine substrate and a graph of fluorescence over 2 hours is shown in Figure 3.24. From this graph it can be seen that fluorescence reaches a peak after 90 minutes, in contrast with the coloured reactions that developed visually between 3 and 4 hours incubation. It was always likely that the fluorescent substrate (Fig 3.23) would produce a more rapid positive reaction than the chromogenic counterpart, as it is widely known fluorogenic substrates are more sensitive than their corresponding chromogenic equivalents (Dealler, 1993). Based upon these experimental results t-boc-val-pro-arg-DMPPD would be a useful substrate for the detection of staphylocoagulase.

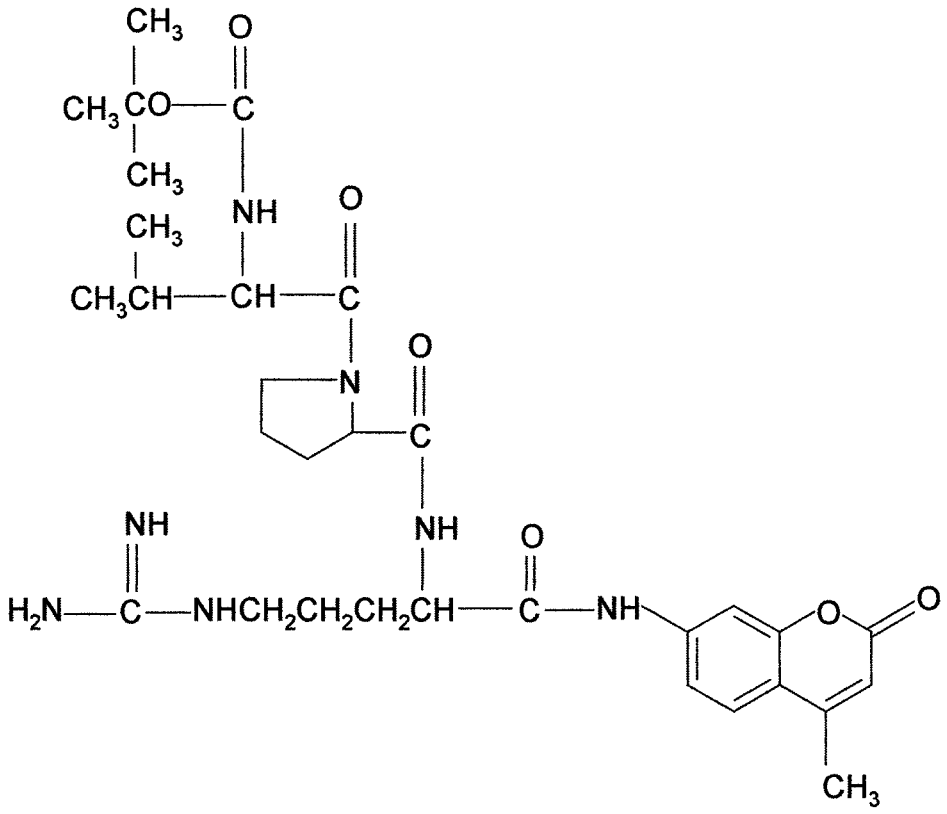


Figure 3.23 Chemical structure of t-boc-val-pro-arg-7-amino-4-methylcoumarin.

Table 3.18. Results observed visually with t-boc-val-pro-arg-DCAP (mg ml^{-1}) in the presence of 13 Staphylococcal and 1 Enterococcal strains

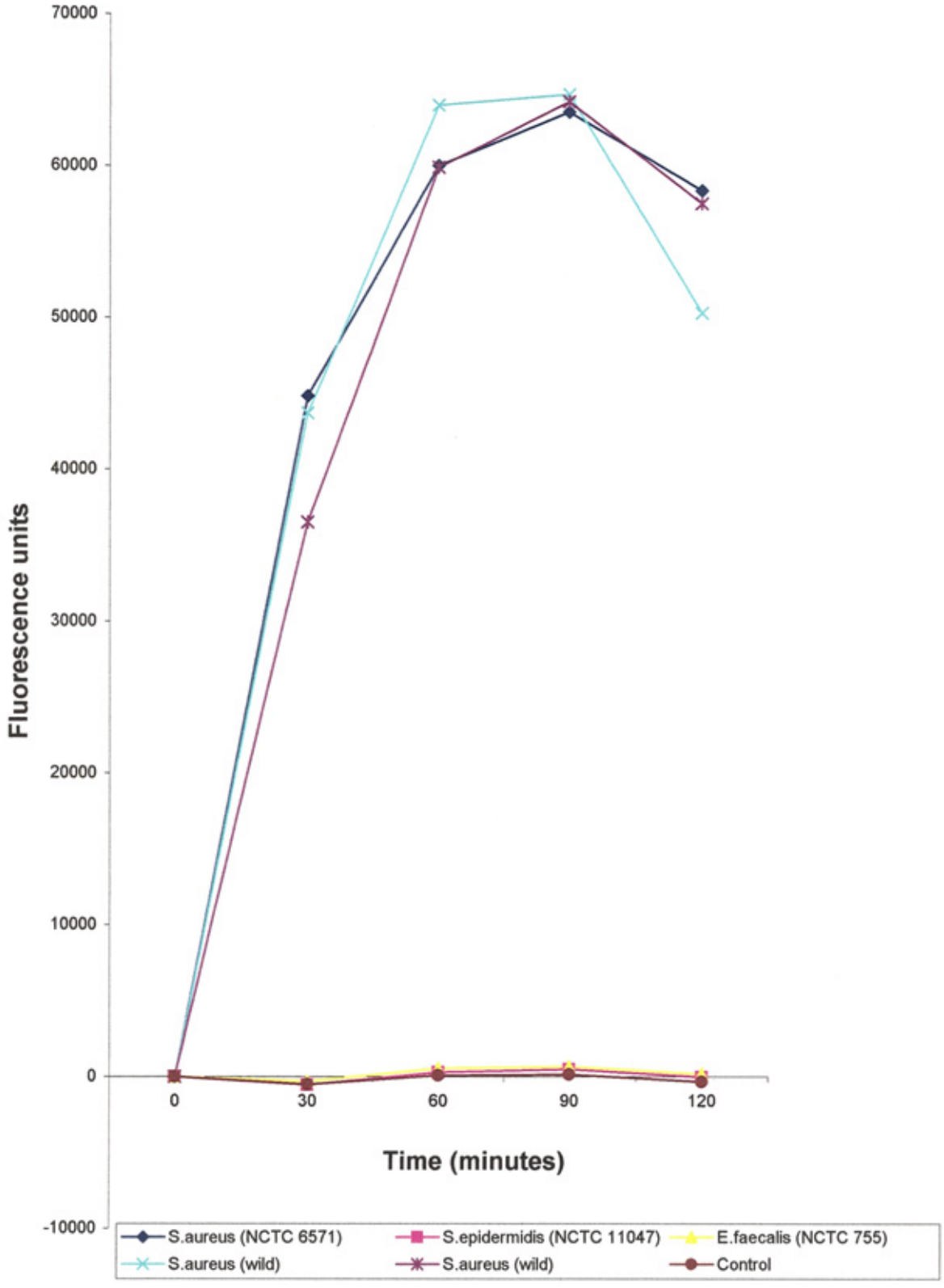
	Concentration (mg ml^{-1})						
	0.012	**0.025**	**0.05**	**0.25**	**0.5**	**1.5**	**2.5**
S.aureus (NCTC 6571)	-	-	-	-	-	+	+
S.epidermidis (NCTC 11047)	-	-	-	-	-	-	-
E.faecalis (NCTC 755)	-	-	-	-	-	-	-
S.aureus (wild)	-	-	-	-	-	+	+
S.aureus (wild)	-	-	-	-	-	+	+
S.aureus (wild)	-	-	-	-	-	+	+
S.aureus (wild)	-	-	-	-	-	+	+
S.aureus (wild)	-	-	-	-	-	+	+
S.aureus (wild)	-	-	-	-	-	+	+
S.aureus (wild)	-	-	-	-	-	+	+
S.aureus (wild)	-	-	-	-	-	+	+
S.aureus (wild)	-	-	-	-	-	+	+
S.aureus (wild)	-	-	-	-	-	+	+
S.aureus (wild)	-	-	-	-	-	+	+
Control	-	-	-	-	-	-	-

Legend: + strongly positive (colured reaction), - negative reaction(no colour)

Table 3.19. Relative fluorescence units obtained with t-boc-val-pro-arg-7AMC (0.25 mg ml^{-1})in the presence of 13 Staphylococcal and 1 Enterococcal strains

	Time (minutes)				
	0	**30**	**60**	**90**	**120**
S.aureus (NCTC 6571)	0	44838	60004	63525	58363
S.epidermidis (NCTC 11047)	0	-544	293	488	-46
E.faecalis (NCTC 755)	0	-305	534	641	168
S.aureus (wild)	0	46389.2	63989	64678	50288
S.aureus (wild)	0	24416	44361	54399	49422
S.aureus (wild)	0	36568.02	53108	63771	50586
S.aureus (wild)	0	33182	43880	60124	54321
S.aureus (wild)	0	31042	57212	65135	57210
S.aureus (wild)	0	37989	59103	66151	58970
S.aureus (wild)	0	29988	58901	66002	53896
S.aureus (wild)	0	31258	49755	57987	50246
S.aureus (wild)	0	45220	56308	71008	61806
S.aureus (wild)	0	27221	57210	63781	57631
S.aureus (wild)	0	36512	59897	64209	57499
Control	0	-522	77	134	-357

Figure 3.24. Fluorescence generated by various staphylococci and *E.faecalis* by hydrolysis of t-boc-val-pro-arg-7-AMC



Discussion

Several novel substrates for phosphatases, glycosidases and peptidases have been synthesised and evaluated for the detection of bacterial enzyme activity in comparison with commercially available conventional chromogenic alternatives. These novel substrates were mainly derivatives of 4-aminophenol, 4-amino-2,6-dichlorophenol, and diethyl-*p*-phenylenediamine as described in Chapter 2. From the results of substrate evaluations in a liquid medium it is clear that most of the novel substrates coupled with naphthol to produce the expected colour, when hydrolysed by the test organisms. In general the corresponding test nitrophenol derivative, or nitroaniline substrate was hydrolysed by more test strains and in many cases produced a reaction which was more rapidly detected than the novel experimental substrates, particularly simple 4-aminophenol substrates. In some cases only the conventional substrate was hydrolysed. There are several possible factors responsible for these findings including:- the affinity for the particular test enzyme for the substrate, the degree of binding, and/or release of the chromogen from the substrate molecule; the presence of potential inhibitors in the reaction mix; and the ease of entry/transport of the substrate into the cell and the subsequent release of the product.

It was determined using purified *E.coli* β-galactosidase that 4-aminophenyl-β-D-galactoside had the highest affinity for the enzyme (0.5 mmol l^{-1}), as determined by K_m, with the halogenated substrate producing a similar value

(0.49 mmol l^{-1}) However the V_{max} value which shows the conversion of the ES complex to E + S was nearly treble that of the halogenated derivative and nearly 30 fold greater than ONPG. In a liquid medium, with β-galactosidase-positive organisms, the halogenated galactosidase substrate performed well with results in complete agreement with ONPG. Possible variations can be predicated based on the K_m results obtained for a range β-galactosidase enzymes obtained from bacteria and fungi towards the substrate ONPG, which indicate a broad range of affinities for ONPG and the different enzymes. Interestingly the K_m for *E.coli* β-galactosidase using ONPG has been determined in previous studies as 0.16 mol l^{-1} (Wallenfels and Malhotra, 1960). The K_m values for β-galactosidases from other species varies widely using the same substrate. For example the K_m for the β-galactosidase of *Thermomyces lanuginosa* is 11.3 mol l^{-1} (Fischer *et al.*, 1993) and 0.04 mol l^{-1} for *Lactobacillus heleveticus* (Nadder de Macias *et al.*, 1983).

The reaction products were easier to read visually than ONPG, as would be expected with a substrate having a V_{max} value more than 10 fold that of ONPG. It is also possible however that the reaction products were easier to read than ONPG due to the strong blue colour produced being more easily differentiated from the background than the yellow colour of *o*-nitrophenol.

The results of the novel substrates were not always in complete agreement with those of the corresponding chromogenic substrate. Using 4-aminophenyl-β-D-xyloside, the nitrophenolic substrate produced reactions in *E.cloacae* and *K.pneumoniae* in approximately 50% less time than the 4-aminophenyl substrate, with *E.faecalis* requiring overnight incubation to produce a positive result. From the results of enzyme kinetics using β-galactosidase, the 4-aminophenol substrate has a higher affinity for β-galactosidase than ONPG and a higher V_{max}, converting 3.49 mmoles of substrate per litre per minute. If the results for the β-galactosidase substrate evaluation tests with the various micro-organisms were extrapolated from the results of enzyme kinetic studies performed for purified *E.coli* β-galactosidase, it would have been expected that the 4-aminophenyl substrates would produce positive results before those obtained with the nitrophenolic equivalent. It is also clear for the results of β-xylosidase activity that the findings obtained for one glycosyl hydrolyase can only be applied to the particular substrate investigated, otherwise the results of studies using 4-aminophenyl-β-D-xyloside would have been expected to have produced the opposite to what was observed experimentally.

The lack of correlation was most evident when β-glucosidase substrates were examined, with 4-aminophenyl-β-D-glucoside failing to produce a positive reaction with known β-glucosidase-positive strains, e.g.

S.marcescens and *K.pneumoniae*. It would suggest that within members of the Enterobacteriaceae, β-glucosidase structure and activity may be different enough to produce negative results with some substrates. This may be due to poor affinity of the enzyme for the substrate, or a very low V_{max} once the enzyme has bound to the active site. This would potentially produce a negative test result with known positive strains. Continued incubation failed to produce any coloured product and suggests enzymes from these strains may have both a high K_m and low V_{max}. The group of enzymes known as exopeptidases catalyse the stepwise cleavage of an amino acid from the amino terminal of a peptide (Asano, 1989). While present in mammalian, bacterial and fungal cells, these enzymes have specificities, which are radically different suggesting an evolutionary divergence (Gasparello-Clemente and Silveira, 2002). It is possible that glycosidase enzymes within individual bacterial species show similar differences in substrate specificities. Alternativly, other factors may be involved in the delivery of substrate to the enzyme and secretion of a coloured reaction product, or an enzyme product which then couples to form the coloured reaction product. Only further detailed research into the hydrolysis of each particular glycosidase substrate by a range of purified enzymes would answer such questions.

The fact that overall the dichlorophenol substrates performed as well as traditionally used nitrophenolic compounds or nitroanilides indicates that

the additional chemical groups fail to hinder the generation of a reaction product in four hours. From the kinetic experiments the dichlorophenol substrate showed a strong affinity for β-galactosidase compared with ONPG, and a higher $V_{max.}$ It is possible that the low V_{max} of ONPG is due to problems in enzyme/substrate recognition (Ito *et al.*, 2000) potentially caused by the effect of the electron withdrawing nitro group para to the site of enzyme action. In these experminents the increased negative-charge produced by the two Cl^- atoms only partially inhibits the formation of a tightly bound ES complex when compared with the non-halide substrate. Studies by Ring and Huber (1993) showed that iodination of *E.coli* β-galactosidase does not affect binding, but causes the enzyme to lose catalytic activity. It is feasble therefore that the presence of chlorine atoms on the substrate may interfere with catalytic activity. However, of the substrates examined in liquid medium, overall the halogenated substrate preformed well, producing visibly strong reactions, often easier to detect than using nitrophenolic derivatives or *p*-nitroanilides.

Glycosyl hydrolases, a family to which β-galactosidase belongs are known to have a modular 3D structure containing a catalytic domain and one or more non-catalytic regions (Jedrzejas, 2000). Some of these non-catalytic regions are involved in substrate binding. The topography of these enzymes shows numerous protein folds, within which are the catalytic sites (Davies and Henrissat, 1995). It is possible that electrostatic bonds occur hindering

the release of certain compounds or the actual hydrolysis of the substrate hence the low V_{max} values. This may explain the low V_{max} values for the ONPG substrate as the negative charge on the released phenol may interfere with substrate release. This is unlikely however since the dichloro substrate is more negatively charged than ONPG and as such if the work of Davies and Henrissat (1995) were applicable to these results the V_{max} value for 4-amino-2,6-dichlorophenol would have been expected to have been much lower. From the data obtained in these experiments however, the results of K_m and V_{max} appear to have little bearing on how the substrate performs experimentally.

The fact that the 4-aminophenol derivative overall performed poorly in liquid media was surprising based on the results of the β-galactosidase experiments. Since both the structure and the active site of glycosyl hydrolyases differ markedly in each of the 45 different major families of enzymes (Jederzejas, 2000), it is conceivable that differences in affinity and ultimately hydrolysis could occur within each bacterial genus for each given test substrate. Indeed it has been shown in *E.coli* that production of β-galactosidase varies depending upon the conditions, and that differences in activity can occur depending upon the environmental conditions (Craig and Hall, 2000). Whilst the environmental conditions of the assay are identical, problems in substrate purity (Craig *et al.*, 2000), or possible toxicity effects may explain some of the discrepant results observed in this Chapter. Other

possible explanations include differences in enzyme affinities, optimal environmental parameters such as pH (Huber *et al.*, 1983) rates of hydrolysis or residence of hydrolysis product within the active site, although the process of how the substrate enters the cell may be of equal importance as the process of hydrolysis.

It is possible that the reason for the discrepant results is related not to problems of affinity or release of the chromogen from the enzyme core but to uptake/diffusion of the substrate into the individual bacterial cells (Sanderman, 1977). Enzymes, such as α and β-galactosidase are present in the cytoplasm of bacteria (Pourcher *et al.*, 1990) and not the periplasm (Bacci Junior *et al.*, 1996). Most large molecular weight compounds such as enzymes and other cell components cannot escape these boundaries, while solutes such as substrates diffuse easily. Many hydrophilic compounds pass through porins of the outer membrane of Gram-negative bacteria. In addition bacteria posess a range of permease enzymes to improve the passage of nutrient compounds into the cell. In the case of metabolism of lactose, a lactose permease, coded by *LacY*, (Brabetz *et al.*, 1993) is required for the rapid uptake of lactose and other similar compounds e.g. ONPG into the cell where cleavage by β-galactosidase then takes place within the cytoplasmic space. The resulting chromogen, e.g. *o*-NP in the case of ONPG, then diffuses easily out of the cell and into the surrounding medium, hence the yellow colour observed with a positive reaction. Indeed,

the strong electron-withdrawing property of the nitro group enhances enzyme hydrolysis rates of nitrophenol based substrates (Haugland, 1996), which would confer an advantage over the substrates tested in these experiments. These substrates offer numerous advantages over a "natural" substrate, being both highly reactive and sufficiently sensitive for the rapid detection of a range of enzyme activity in microorganisms (James, 1994). However it has been shown that the substrate *p*-nitrophenyl-α-maltoside is transported into the cell (Reyes *et al.*, 1986)

Based on finding that nitrophenolic substrates diffuse rapidly into the cell and undergo rapid hydrolysis due to the electron withdrawing properties of the NO_2 group (Haugland, 1996), it is important to consider if these novel substrates also enter the cell in the same way. One possible explanation for the discrepant findings in this chapter, may relate to the diffusion of these novel chromogenic compounds into the bacterial cells, or the ability of individual permease enzymes to facilitate the transfer across the cell membrane. Studies have shown that the lactose permease enzyme differs markedly between bacterial species (Leong-Morgenthaler *et al.*, 1991). Interestingly ONPG does not require a permease to facilitate cell entry (James, 1994).

Studies using *S.sonnei*, have found that the slow lactose fermentation associated with this organism was associated with weak lactose permease

activity, which is much lower than that observed in *E.coli* K12 (Kido *et al.*, 1991). Whilst the mechanisms of transportation of lactose into the cell is widely known, how novel substrates such as aminophenols and *p*-phenylenediamines are transported into the cell is unknown. It is conceivable that two problems at a cellular level exist with the transport or release of the cleaved products back into the surrounding medium. For example there were variations in the reactions obtained between 4-aminophenyl-β-xyloside and *o*-nitrophenyl-β-D-xyloside with *E.cloacae, K.pneumoniae* and *E.faecalis*, with the nitrophenolic substrate producing a visibly faster reaction than the conventional test substrate. Based on the strong results obtained with nitrophenolic substrates and the dichloro substrate, it is possible that the halogen atoms act in the same way as the nitro group and facilitate entry of the substrate into the cell. It is conceivable that the often delayed or negative reactions obtained with simple 4-aminophenol substrates may be due to poorer diffusion of the 4-aminophenol into the cell, or to problems with diffusion back into the surrounding medium following hydrolysis. It is known that natural substrates such as xylose require permease enzymes to facilitate rapid cell entry. Uptake of xylose (Hamacher *et al.*, 2002) has been shown in yeast cells (*Saccharomyces cerevisiae*) to require a number of specific xylose transporting proteins coded as Hxt 4, Hxt 5, Hxt7, and Gal2. It is possible that in the test strains, the loss of one or more of such transporting proteins would lengthen the time for a positive test result to occur or indeed not to

occur at all. It is also possible that these transporter proteins may be required to uptake certain aminophenolic substrates into the cell and the lack of these proteins or poor affinity for the substrate would explain the poor results obtained using substrates based on simple 4-aminophenol. Only further research in this area would reveal if the poor results were the result of poor uptake or secretion from the cell. However, the fact that different bacteria may have dissimilar transport systems for a particular substrate, offers another feasible explanation of differences in test results between the various novel substrates.

Few new novel enzyme substrates for use in liquid media have been developed, despite problems that exist with current chromogenic and fluorogenic substrates. It has been shown here that glycosidase substrates of 4-aminophenol, particularly the halogenated derivative could be useful as alternatives to nitrophenol substrates. In addition the present study has demonstrated that aminopeptidase substrates of 4-aminophenols and *p*-phenylenediamines show promise for the detection of this group of enzymes in a range or bacteria. Substrates based on 4-aminophenol, have a major advantage over *p*-phenylenediamine substrates, which can be derivitised at both the amino and hydroxyl positions to produce substrates for glycosidases and aminopeptidases. The main disadvantages of currently available substrates, particularly those based on 7-AMC, 4-MeU, nitrophenol and nitroaniline, is that the released chromogen or fluorogen

diffuses widely through agar. It is hoped that these novel substrates will show potential for use in solid media, either as presented in this chapter or by derivitisation to enhance localisation on individual bacterial colonies. The results of experiments with these substrates in solid media are evaluated in Chapter 4.

Chapter 4

Evaluation of substrates based on *p*-phenylenediamines and 4-aminophenol for the detection of bacterial hydrolyases in solid media.

Introduction

In medical microbiology, the identification of target organisms generally requires a combination of microscopy, serology, and more recently molecular methods e.g. real time PCR (Ke and Bergeron, 2001). Traditional culture methods and subsequent use of a chromogenic or fluorogenic substrate still remains the gold standard in bacterial identification (Houpikian and Raoult, 2002). Ideally, the substrate would be incorporated into the primary isolation medium and would identify the target organism without the requirement for an additional identification step. As discussed in Chapter 1 numerous substrates are available for the detection of organisms in liquid media, but many of these cannot be used in solid media due to widespread diffusion of the released chromogen (Manafi, 1991). Thus, target organisms in mixed culture are impossible to differentiate and identify correctly using such substrates.

Diffusion through solid media is affected by a number of factors, most notably composition, depth of the medium, and pH (Garrod *et al.*, 1981). Perhaps the most important factor in diffusion of a compound through a solid medium is the chemical composition of the test compound. Whilst no quantitative studies have been performed on how released chromogens or fluorogens diffuse through agar, comparisons can be made with antibiotics, compounds which have been regularly examined in solid media, e.g.

Colimycin, (polymixin E) is a high molecular weight polypeptide antibiotic, and such a high molecular weight compound, with long hydrophobic side chains would be expected to diffuse poorly through an aqueous medium solidified with agar (Kunin and Edmondson, 1968). Indeed this has been observed in disk sensitivity testing with this compound. Small molecular weight compounds with polar substituent groups such as hydroxyl and carboxylic acid groups, are often highly soluble in water, and thus are likely to rapidly diffuse through agar (Figure 4.1). This explains the limited use of substrates of *o*-NP and *p*-NP and well as coumarins in solid media (Manafi, 1991).

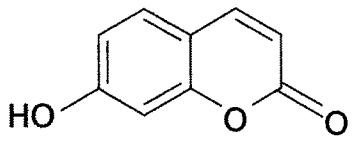


Figure 4.1 Chemical structure of a simple coumarin (7-hydroxycoumarin).

A different approach is thus required for the production of substrates which remain highly localised on individual bacterial colonies upon enzyme hydrolysis. Early studies on the development of chromogenic substrates, which could be used in solid media, were based upon the principle of chelation with metal ions. Most substrate-chelate interactions in microbiology are formed using iron (III) compounds, the most widely used of which is ferric ammonium citrate (Perry *et al.*, 2000). While other metal

ions can be used to form such chelates, many can be toxic to bacterial enzymes e.g. copper (Sterritt and Lester, 1980; Keeling and Cater, 1998). Iron (III) is ideally suited to chelate formation because of its high charge density due to its tri-positive charge (Porter *et al.*, 1989). It thus interacts strongly with polar groups bearing a similarly high negative charge density. This state occurs in compounds containing oxygen atoms, particularly carboxylic acids, catecholates and hydroxypyridinones e.g. Figure 4.2.

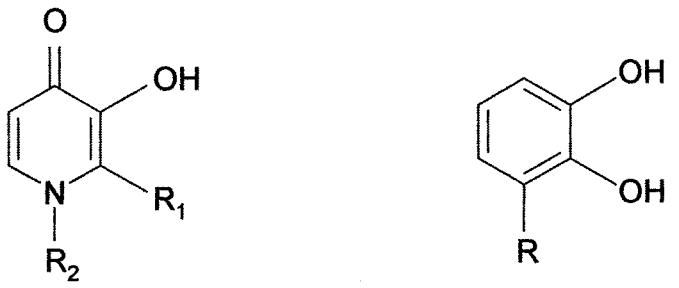


Figure 4.2 Structures of a potential hydroxypyridinone (left) and catecholate.

The stability of the complex is influenced by the number of covalently linked ligands on the chelator, with hexadentate ligands being more stable than bidentate ligands (Porter *et al.*, 1989). For example, desferrioxamine is a hexadentate ligand and its extreme affinity for iron makes it useful *in vivo* as a treatment for patients with iron overload (Link *et al.*, 2001).

The first widely used substrate that utilised chelation for detection of the reaction product was aesculin (6,7-dihydroxycoumarin-β-D-glucose), derived from the horse chestnut tree (*Aesculus hippocastanum*), and a compound that is also naturally fluorescent (Edberg *et al.*, 1976). The compound possesses important functional groups which would be expected to give rise to metal chelates, particularly the hydroxyl groups at positions 6 and 7 on the coumarin ring (Figure 4.3). When hydrolysed by the enzyme β-glucosidase, a brown-black complex is immediately formed in the presence of ferric ions (Trepeta and Edburg, 1987). The compound is commonly used in diagnostic tests for the differentiation of *Enterobacteriaceae* (Edberg, 1977), and also for the detection of enterococci in water samples (Daoust and Litsky, 1975; Knudtson and Hartman, 1992).

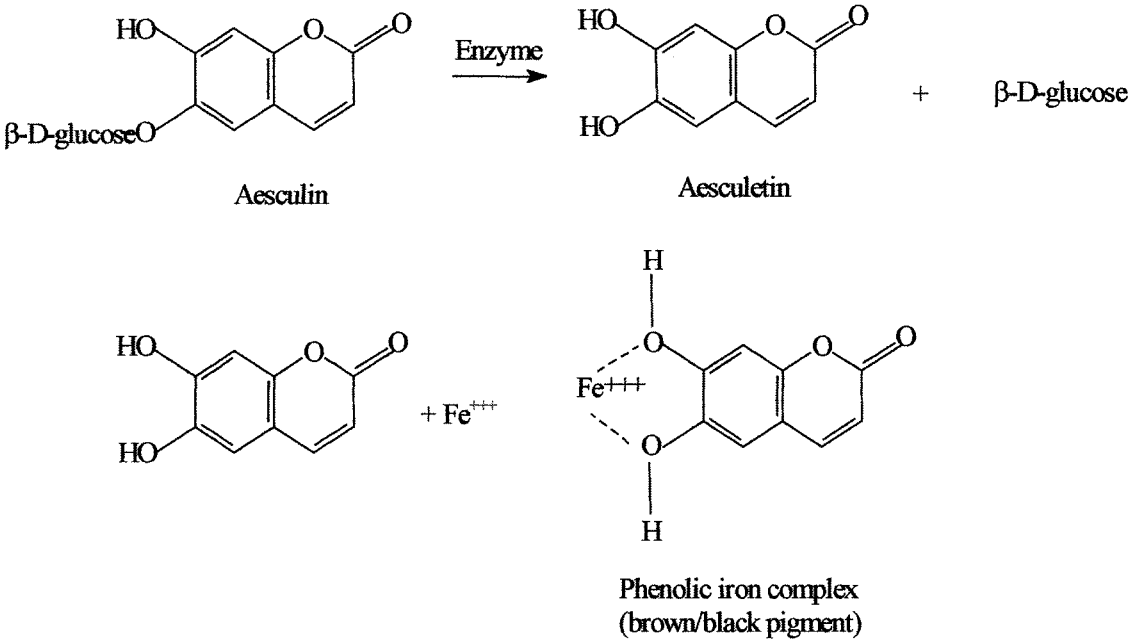


Figure 4.3. Chemical structure of Aesculin and the metal complex formed upon hydrolysis by β-glucosidase in the presence of ferric ions (MacFaddin, 1980).

The brown-black complex formed is a large hydrophobic metal chelate, which being water-repelling, therefore, diffuses slowly through solid media (Albert, 1953). Despite aesculin forming a metal chelate with iron (III) upon hydrolysis with β-glucosidase, the brown-black precipitate diffuses to a limited extent through solid media making bacterial colony counting of aesculin-hydrolysing bacteria difficult if not impossible in mixed cultures. This is especially true when large numbers of organisms are present (Brodsky and Schiemann, 1976). This problem has prompted the

development of other substrates that upon hydrolysis also form metal chelates, yet remain more highly localised on individual bacterial colonies.

In two publications by James and Yeoman (1987a and 1987b) substrates were developed for the detection of bacterial β-glucosidase and β-glucuronidase respectively using 8-hydroxyquinoline derivatives. When applied to the detection of β-glucosidase, a highly localised, strongly lipophilic, black pigment was observed (Figure 4.4).

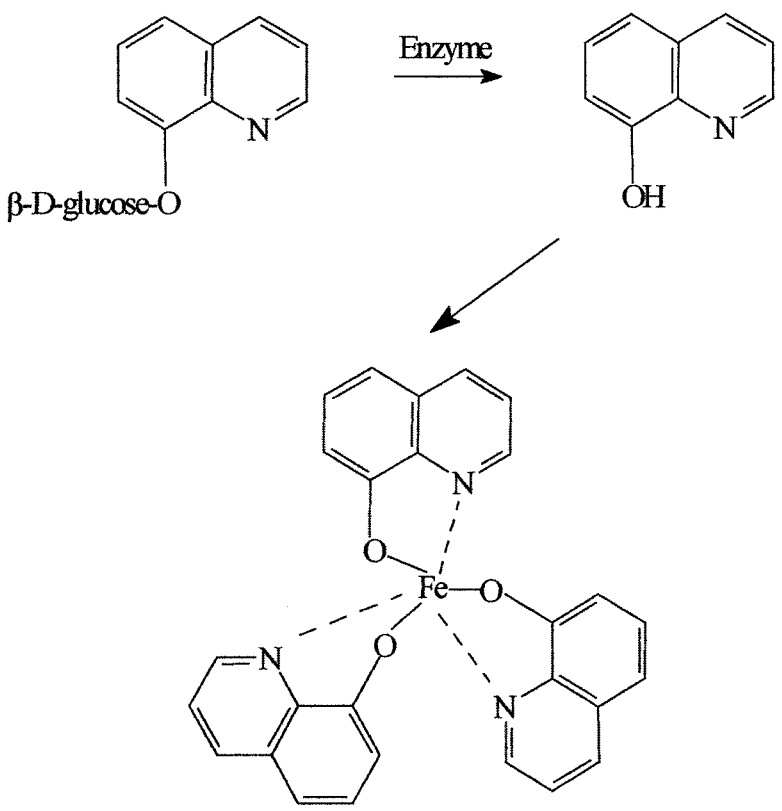


Figure 4.4. Chemical structure of 8-hydroxyquinoline-β-D-glucoside and the complex formed upon chelation with iron.

This substrate permitted the easy detection of β-glucosidase producing strains. The detection of β-glucuronidase activity in *E.coli* was however disappointing using 8-hydroxyquinoline-β-D-glucuronide. In addition, 8-hydroxyquinoline has been shown to be toxic, particularly towards Gram-positive organisms (Albert *et al.*, 1953). The toxic effect is augmented by the presence of the chelating iron. This metal and 8-hydroxyquinoline form a lethal complex, even when the concentration of the iron is not toxic. However, this toxic effect can be antagonised by cobalt, cadmium and nickel (Sterritt and Lester, 1980). Thus, whilst remaining highly localised,

substrates of 8-hydroxyquinoline are not ideal for use in routine isolation media.

More recently a newer compound, cyclohexenoesculetin (James, 1997) has been developed for the detection of glycosidase activity (Fig 4.5).

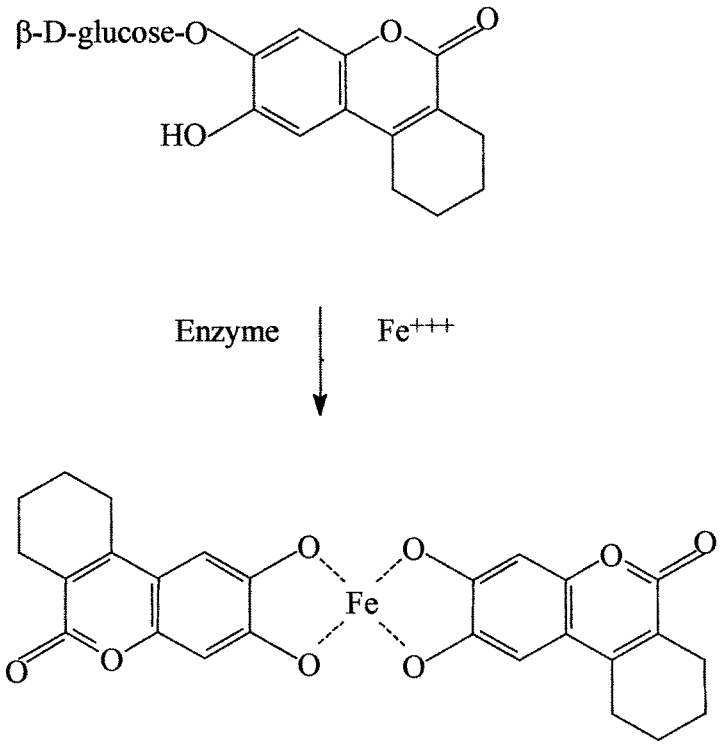


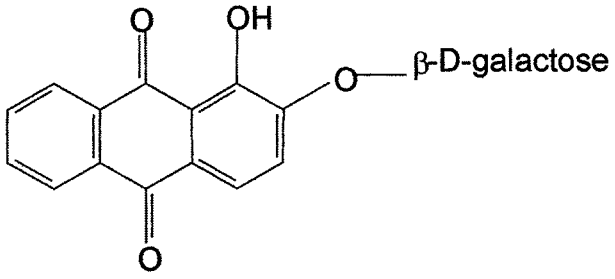
Figure 4.5. Structural formula for cyclohexeneoesculetin-β-D-glucoside, and the complex formed with iron following enzyme hydrolysis.

When used for the detection of β-galactosidase activity in Enterobacteriaceae all β-galctosidase positive strains hydrolysed the substrate to produce highly localised black colonies in the presence of ferric

ions. In comparison, 8-hydroxyquinoline-β-D-galactoside was only hydrolysed by 56% of the Enterobacteriaceae strains tested (James, 1997).

Perhaps one of the most interesting compounds recently developed for use in solid media is alizarin (1,2-dihydroxyanthraquinone), which is one of the most important anthraquinoid mordant dyes, forming different colours depending on the metal salt used (Finar, 1975). When derivitised at position 2 on the anthracine ring to produce substrates for glycosidases (Figure 4.6), enzyme hydrolysis yields a highly localised coloured reaction product.

(a)



(b)

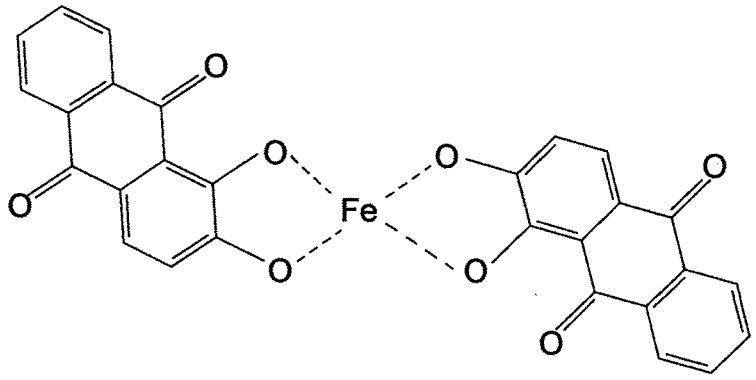


Figure 4.6. Structure of alizarin-β-D-galactoside showing the complex formed following enzyme hydrolysis. (a) alizarin-β-D-galactoside; (b) complex formed upon chelation of the released alizarin with iron (James *et al.*, 2000).

The colour of the complex depends upon the metal ion used for chelation (James *et al.*, 2000). In the presence of ferric ions, a violet-black colour is observed, and aluminium yields a bright red pigment. It is possible that alizarin substrates will be widely used for the detection of glycosidases, due to both ease of synthesis, and to the low concentration required for formation of the coloured product (James *et al.*, 2000). Such properties

make alizarin substrates ideal for the routine detection of glycosidase activity in solid media.

Despite these recent advances, indoxyl substrates (Fig 4.7) are still the most widely used for the detection of glycosidase activity in solid media. These widely available substrates undergo rapid air oxidation upon hydrolysis to form a bright blue, highly stable indigo dye (Kodaka, 1995). Derivatisation by the addition of halogen atoms to the benzyl ring of indoxyl has allowed the production of substrates which form different coloured indigo dyes upon oxidation.

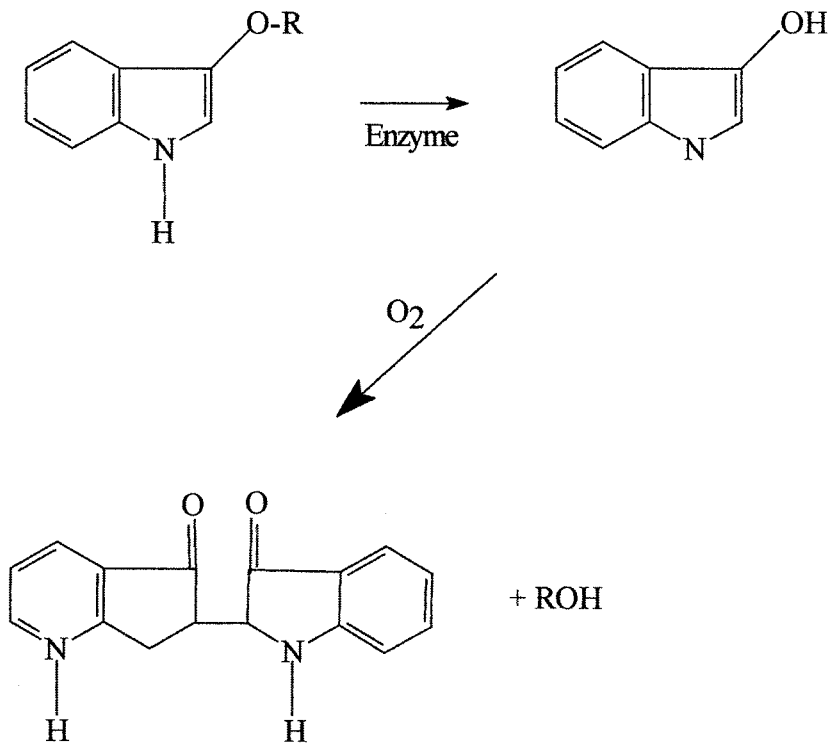


Figure 4.7. Diagram showing the structure formed of an indoxyl derivative following enzyme hydrolysis and reaction with atmospheric oxygen.

Several authors have utilised these indoxyl substrates for the detection of enzymes in tissue (Pearson, 1967) and for detection of specific microorganisms in solid media (Haines *et al.*, 1993; Jabra-Rizk *et al.*, 2001). A major advantage of using substrates whose products are different colours upon hydrolysis, is that several different substrates can be incorporated into the same medium allowing the identification of several target bacterial species using a single diagnostic medium. Indeed several commercially-produced media utilise this approach. A review by Manafi (1996) describes the use of various indoxyl substrates in solid media.

Indoxyl, cyclohexenoesculetin, and 8-hydroxyquinoline substrates have several key disadvantages. Synthesis of these substrates is complex, and this is reflected in the purchase cost, especially for halogenated indoxyl derivatives. However, increasing use, to a certain extent, has enhanced their cost-effectiveness. Secondly, a high concentration is required for use in solid media as compared to liquid studies that are often performed using very small volumes. In addition, these substrates are only available for the detection of glycosidase activity: an aminopeptidase substrate that remains localised on the colony mass has yet to be developed. No studies have yet examined the toxicity of CHE or indoxyl compounds, but 8-hydroxyquinoline is highly toxic towards several bacterial groups, most notably Gram-positive organisms (Albert *et al.*, 1953).

This chapter discusses the use of derivatised 4-aminophenols and *p*-phenylenediamines in solid media as alternatives to indoxyl, cyclohexeneoesculetin and alizarin substrates. Based on the chemical structure of indophenol blue (Figure 1.4) it is unlikely that the compound formed will undergo chelation with metal ions, since functional groups, which promote chelate formation, are absent. Further derivatisation may increase both the lipophilic and hydrophobic nature of the substrate, or functional groups added, which may promote chelation. Alternatively increasing the molecular weight of the complex may prove useful in limiting any potential diffusion, and this could be achieved by the addition of side

chains to either the core compound or the coupling naphthoic derivatives. Finally the use of molecules based on deoxycholic acids and related compounds have been examined as a method for limiting the diffusion of the coloured product through agar.

Materials and Methods

Growth media

All organisms used were subcultured onto Columbia blood base (Oxoid) prepared as described previously (Chapter 2). The organisms were incubated at 37°C overnight and checked for purity before use. For initial screening of substrates, naphthols and media containing "blocking" agents, the Gram-negative control organisms used were chosen from *E.coli* (NCTC 10418), *Klebsiella pneumoniae* (NCTC 10896), *Enterobacter cloacae* (NCTC 11936), *Serratia marcescens* (NCTC 10211), *Salmonella typhimurium* (NCTC 74). The Gram-positive control strains used were *Enterococcus faecalis* (NCTC 755), *Staphylococcus aureus* (NCTC 6571).

Preparation of agar plates.

All test compounds were weighed out as required using a Sartorius 2434 electronic balance, accurate to 0.1 mg. (Sartorius Limited, Epsom, UK), and placed into a 100 ml Duran bottle containing the required amount of Columbia agar base (Oxoid). A 100 ml volume of sterile de-ionised water was then added. The ingredients were then mixed to dissolve and the pH adjusted to 7.2 (Hanna Instruments Limited, Leighton Buzzard, UK). Each medium was then sterilised by autoclave at 114°C for 10 minutes, then

placed in a water-bath (Grant instruments, Cambridge, U.K) and cooled to 50°C. The cooled media were then poured into sterile 90 mm petri dishes (RossLab, Macclesfield, U.K) and allowed to set. Before inoculation the plates were incubated in a 37°C incubator (Laboratory and Electrical Engineering Company, Nottingham, U.K) overnight as a sterility check. Deoxycholate citrate agar (Lab M, Bury, U.K) was prepared according to manufacturer's instructions and sterilised and poured as described above.

Preparation of inoculum

Using a 1µl loop a small portion of a well-isolated single colony was emulsified in 2 ml of sterile API buffer, and adjusted to a McFarland standard 0.5 using an API Densimat (BioMerieux, 69280 Marcy – l'Etoile, France). The suspension was further diluted by 10^3 in API buffer and 10 µl applied to the surface of each agar plate using a sterile plastic loop (Sarstedt, Boston Road, Leicester, UK) and spread for single colonies. This was the equivalent of applying approximately 10^4 CFU of each organism to the surface of the medium. For experiments requiring the use of multi-point inoculation, the suspension was diluted by 10^2 in API buffer and 1µl applied to the surface of each plate using a multi-point inoculator (Denley Instruments, Billingshurst, West Sussex, U.K). Plates were incubated at 37°C overnight and examined for growth and a coloured reaction product.

Chemicals

The following substrates and chemicals were obtained from Sigma Aldrich Chemical Company Ltd, Poole, UK: 3,5-dihydroxy-2-naphthoic acid, ferric ammonium citrate, 8-hydroxycoumarin, 2-aminophenol, phytic acid, 1,5-naphthalenedisulfonic acid (tetrahydrate), taurocholic acid, N-(1-naphthyl)ethylenediamine, taurodeoxycholic acid, glycodeoxycholic acid, dioctyl sulfosuccinate, deoxycholic acid, camphor-sulfonic acid, Sephadex G-25, DEAE-Sephacel, DEAE-Cellulose, poly (acrylic acid), bovine albumin and Kaolin. Irgasan (5-chloro-2-(2,4-dichlorophenoxy) phenol) was obtained from Ciba Speciality Chemicals Ltd, Macclesfield, Cheshire, UK. All novel chromogenic substrates were synthesised by Dr James, along with a sample of N-acetonidophenyl-ethyl-1-hydroxy-2-naphthamide.

Methods

Determination of the optimal concentration of both L-ala-DEPPD and L-ala-DCAP in combination with 3,5-dihydroxy-2-naphthoic acid for use in solid media.

A range of concentrations of L-alanyl-diethyl-*p*-phenylenediamine, L-alanyl-4-amino-2,6-dichlorophenol and 3,5-dihydroxy-2-naphthoic acid were prepared at 5 - 0.625 mmol l^{-1} in doubling dilutions in 20 ml volumes of molten Columbia agar base. Each substrate was examined in a "chequerboard" format against 3,5-dihydroxy-2-naphthoic acid. In addition substrate-free and naphthol-free control plates were also prepared in 20 ml of Columbia agar base as described previously.

A suspension of each organism was prepared as described previously, and then multipoint inoculated onto the agar plates. The plates were incubated at 37°C overnight and examined for the formation of a coloured product and the localisation of colour on the colony mass. Colour intensity was graded visually and the average zone of diffusion for all Gram-negative strains was measured.

Determination of the toxicity of a medium containing optimal substrate and naphthol concentrations.

Two separate 200 ml volumes of Columbia agar were prepared, the first containing 134.4 mg of L-ala-DEPPD and 120.1 mg of 3,5-dihydroxy-2-naphthoic acid. The second contained, 133.1 mg of L-alanyl-4-amino-2,6-dichlorophenol, and 120.1 mg of 3,5-dihydroxy-2-naphthoic acid. This produced a concentration of all reactants of 2.5 mmol l^{-1}, a concentration derived from optimal concentrations obtained in Chapter 2. The medium was sterilised and prepared as described previously.

A 0.5 McFarland suspension of *S.marcescens* and *E.faecalis* was prepared as described previously and then subjected to serial decimal dilution down to 10^{-8} in API buffer. A 50µl volume of each dilution was applied using a sterile Gilsson pipette, to each half of the test medium. As a control Columbia agar without naphthol or substrate was used. All plates were incubated overnight and the colonies counted for each organism using the Gel-Doc Image analyser and Quantity One software (BioRad Ltd, Hemel Hempstead, U.K).

Evaluation of L-ala-4-aminophenol, L-alanyl-4-amino-2,6-dichlorophenol and L-alanyl-DEPPD in the presence of 3,5-dihydroxy-2-naphthoic acid.

To each of three separate 100 ml volumes of Columbia agar base 5.1 mg of 3,5-dihydroxy-2-naphthoic acid were added. To the separate volumes, the following substrates were added: 58.4 mg of L-ala-4-aminophenol, 67.2 mg of L-ala-DEPPD and 66.5 mg of L-ala-DCAP. This produced final substrate and naphthol concentrations of 2.5 mmol l^{-1}. A 2.5 mmol l^{-1} solution of 3,5-dihydroxy-2-naphthoic acid was prepared by dissolving 51 mg in 1 ml of 0.1 mol l^{-1} NaOH followed by 99 ml of sterile de-ionised water. The pH was adjusted to 7.2 upon dissolution. In addition media were prepared containing L-ala-DEPPD without naphthol and L-ala-4-aminophenol, again without naphthol. The media were then mixed, sterilised and poured as described previously.

A suspension of each organism was prepared as described previously and 10 µl placed onto the test media, and spread for single colonies. The plates were incubated at 37°C overnight. Following incubation, the plates containing substrate only were flooded with a 2.5 mmol l^{-1} solution of 3,5-dihydroxy-2-naphthoic acid, the excess removed, and incubated for 30 minutes at 37°C. All plates were examined for the formation of a coloured

product and the localisation of colour on the colony mass. An un-inoculated control plate was also examined to check on substrate stability.

Evaluation of the effect of the chelating agent ferric ammonium citrate on diffusion of the coloured reaction product in solid media containing L-ala-DEPPD or L-ala-DCAP in the presence of 3,5-dihydroxy-2-naphthoic acid.

Five separate 20-ml volumes of Columbia agar were prepared containing 13.4 mg of L-alanyl-DEPPD and 12 mg of 3,5-dihydroxy-2-naphthoic acid. Ferric ammonium citrate was then added at concentrations of 0, 12.5, 25, 50 or 100 mg l^{-1}. This was repeated with the L-ala-DEPPD replaced by 13.3 mg of L-ala-DCAP. The media were sterilised and poured as described previously.

A McFarland 0.5 standard was prepared for all test organisms, and this was diluted by 10^3 using sterile API buffer. The test organisms were multi-point inoculated onto the media as described previously. The plates were incubated overnight at 37°C and examined visually for both intensity and diffusion of the coloured reaction product. The zones of diffusion were measured using the Gel-Doc Image analyser and Quantity One software (Biorad Ltd, Hemel Hempstead, U.K).

Evaluation of L-alanyl substrates based on 4-dimethylaminoaniline (N,N-dimethyl-*p*-phenylenediamine) as potential non-diffusible substrates in solid media.

The chemical structures of five L-alanyl derivitised extended *p*-phenylenediamines are shown in Figure 4.8. Compound (a) is a N-(4-alanylamidophenyl)-N-methylaminomethyl-1,3-dioxolane derivative, and compound (b) is N-(4-alanylamindophenyl)-N-methylaminobutyryl-2-napthalenesulphonylhydrazine, a naphthalenesulphonhydrazine derivative of *p*-phenylenediamine. Substrate (c) is 4,4-dialanylamidodiphenylamine the di-alanyl derivative of 4,4-diaminodiphenylamine. Compound (d) is 2,4-diamino-4-methoxydiphenylamine, which is a variant of the redox dye Variamine blue. This dye is not stable in the oxidised (coloured) form, however the 2-amino derivative is highly stable. The derivative was therefore dialaninated to give the test substrate (James, personal communication). Finally, substrate (e) was the result of a condensation reaction of DEPPD and 4-fluoronitrobenzene, yielding 4-nitro-4-diethylaminophenylamine. Reduction of the amine and derivatisation with L-alanine produced the aminopeptidase substrates shown.

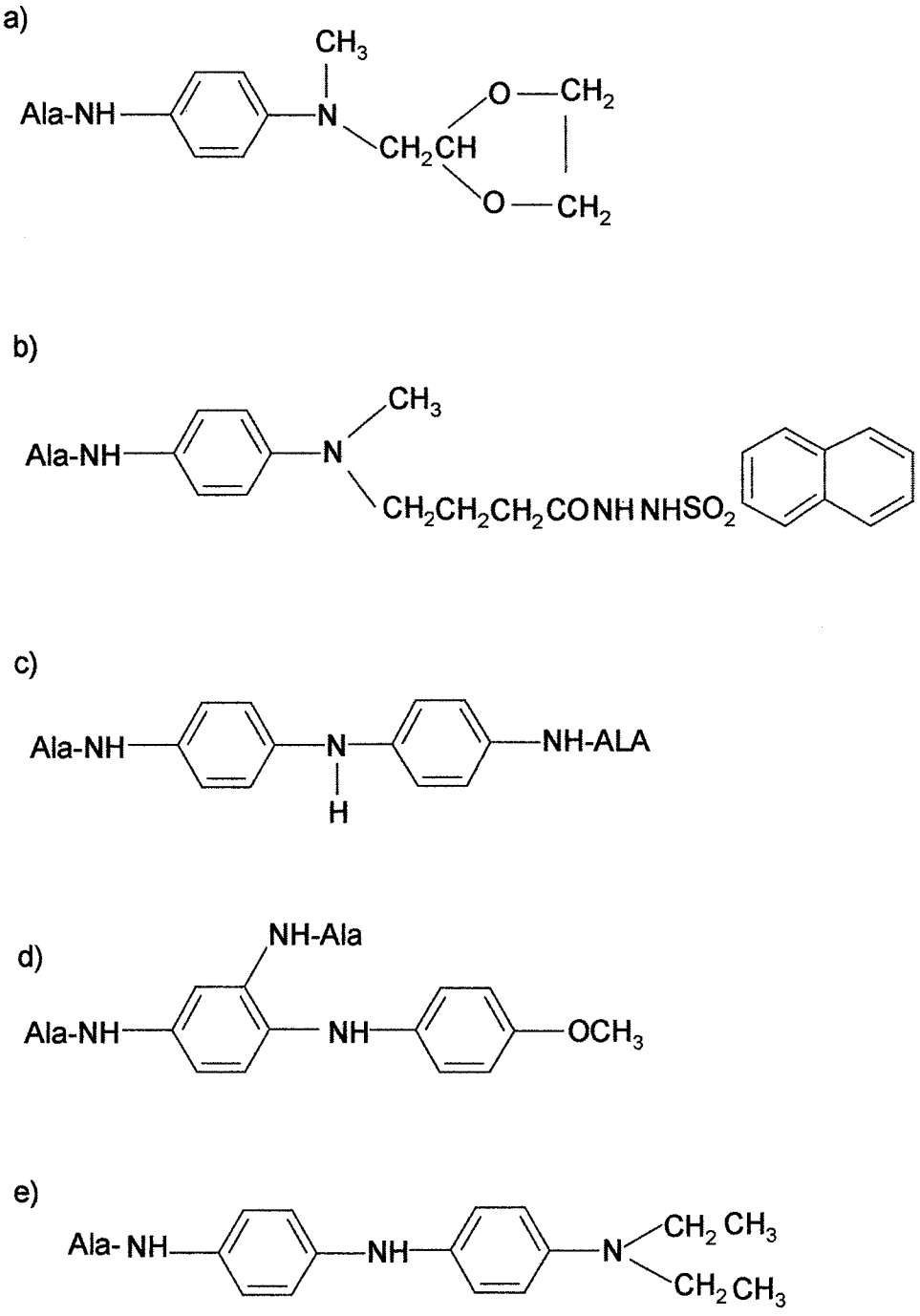
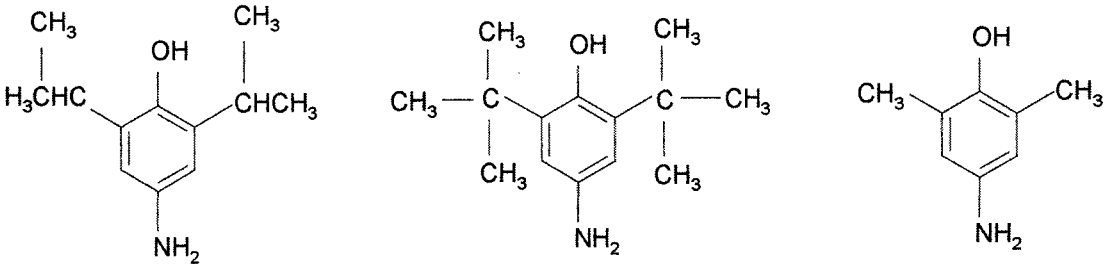


Figure 4.8. Chemical structures of alanyl derivatives of (a) N-(4-alanylamidophenyl)-N-methylaminomethyl-1,3-dioxolane, (b) N-(4-alanylamindophenyl)-N-methylaminobutyryl-2-napthalenesulphonylhydrazine, (c) 4,4-dialanylamidodiphenylamine, (d) 2,4-dialanylamido-4'-methoxydiphenylamine, (e) 4-alanylamido-4'-diethylaminodiphenylamine.

Five separate 100 ml volumes of Columbia agar base were prepared and 52.5 mg of N-(4-alanylamidophenyl)-N-methylaminomethyl-1,3-dioxolane, 103 mg of N-(4-alanylamindophenyl)-N-methylaminobutyryl-2-napthalenesulphonylhydrazine, 93 mg of 4,4-dialanylamidodiphenylamine, 101 mg of 2,4-dialanylamido-4'-methoxydiphenylamine, and 93 mg of 4-alanylamido-4'-diethylaminodiphenylamine were added, one to each 100 ml volume. This produced a final substrate concentration of 2.5 mmol l^{-1}. In addition 5.1 mg of 3,5-dihydroxy-2-naphthoic acid was also added to all volumes. The media were mixed and sterilised by autoclave at 114°C for 20 minutes. The media was then cooled to 50°C in a water-bath and then poured into sterile 90 mm petri dishes. Plates were incubated overnight as a sterility check. After incubation the plates were inoculated with the test organisms as described previously.

Examination of colour produced by L-alanyl derivatives of three substituted 4-aminophenols in solid media.

The three substituted L-alanyl derivatives were: L-alanyl-4-amino-2,6-di-isopropyl-phenol, L-alanyl-4-amino-2,6-di-tertiary-butyl-phenol and L-alanyl-4-amino-2,6-dimethyl-phenol, the underivatised structures of which are shown in Figure 4.9.



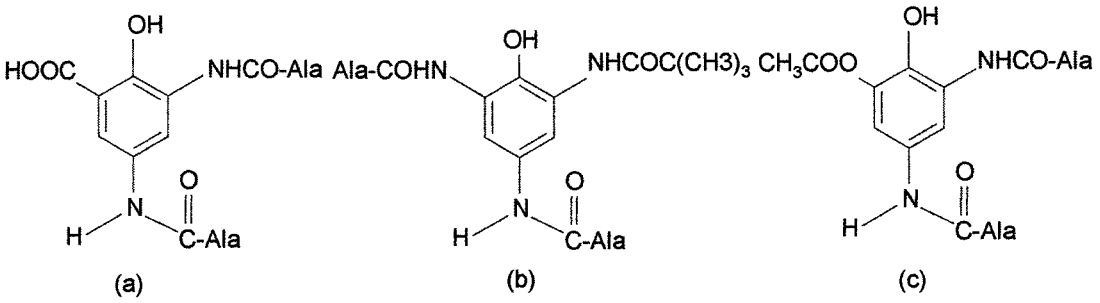
(a) (b) (c)

Figures 4.9. Chemical structures of (a) L-alanyl-4-amino-2,6-di-isopropyl-phenol, (b) L-alanyl-4-amino-2,6-di-tertiary-butyl-phenol and (c) L-alanyl-4-amino-2,6-dimethyl-phenol.

These substrates were examined for colour production in solid media in association with 3,5-dihydroxy-2-naphthoic acid prepared as described previously.

Evaluation of three extended L-alanyl-4-aminophenol substrates in solid media.

Three extended chain L-alanyl-4-aminophenols were synthesised and examined in solid media. The chemical structures of the three extended 4-aminophenols are shown in Figures 4.10. Due to the instability of the compounds in a single L-alanyl form, the di-alanyl compound was produced. Three separate 100 ml volumes of Columbia agar base were prepared containing 99 mg of 1,3-dialanylamido-2-hydroxy-5-benzoic acid, 113 mg of 3,5-dialanyl-amido-2-trimethylacetamido-phenol, and 103 mg of 2,4-dialanyl-amido-6-acetoxyphenol. The media were mixed to dissolve, sterilised, and poured as described previously. An un-inoculated control plate was also examined to check on substrate stability.



Figures 4.10. Chemical structures of (a) 1,3-dialanylamido-2-hydroxy-5-benzoic acid, (b) 3,5-dialanyl-amido-2-trimethylacetamido-phenol and (c) 2,4-dialanyl-amido-6-acetoxyphenol.

Evaluation of L-alanyl-N-phenyl-*p*-phenylenediamine in solid media for the formation of an Indamine complex.

L-alanyl-N-phenyl-*p*-phenylenediamine (Figure 4.11) was prepared at 2.5 mmol l^{-1} by dissolving 46 mg in 100 ml of Columbia agar base. The medium was sterilised and poured as described previously.

A suspension of each organism was prepared as described previously and 10 µl placed onto the surface of the test agar and spread for single colonies. The plates were incubated at 37°C overnight and examined for the production of a coloured product and the localisation of colour on the colony mass. An un-inoculated control plate was also examined to check on substrate stability.

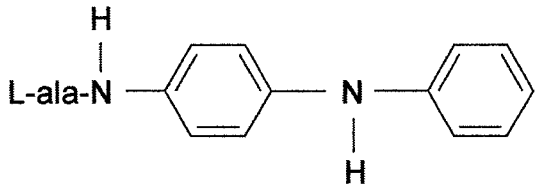
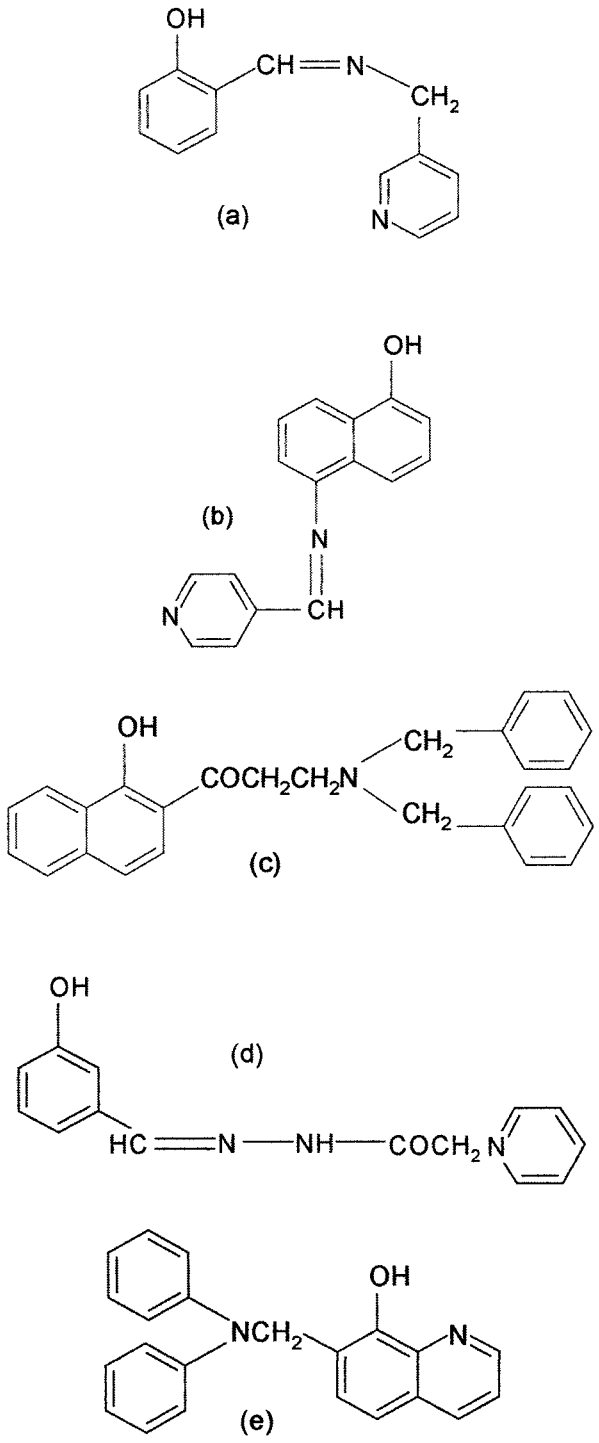


Figure 4.11. Chemical structure of L-alanyl-N-phenyl-1,4-phenylenediamine.

Evaluation of a range of compounds as alternatives to naphthol for the formation of coloured complexes with L-alanyl-DEPPD in solid media.

To 100 ml of Columbia agar, 67.2 mg of L-alanyl DEPPD was added to produce a concentration of 2.5 mmol l^{-1}. Each test compound was also added at a final concentration of 2.5 mmol l^{-1}, this consisted of 35.6 mg of N-salicylidene-3-aminomethylpyridine, 34.4 mg of 1-hydroxy-5-(4-pyridyl-methyl)-iminonaphthalene, 54.8 mg of 2-(N,N-dibenzylaminopropionyl)-1-naphthol, 40.4 mg of 3-hydroxybenzaldehyde-1-(carboxymethyl) pyridinium chloride hydrazone, and 43.2 mg of 7-(N,N-diphenylaminomethyl)-8-hydroxyquinoline (Figures 4.12 a-e). L-alanyl-DEPPD and 3,5-dihydroxy-2-naphthoic acid was used as a control. Each medium was prepared and poured as described previously. Plates were inoculated with NCTC strains as described previously. Plates were examined after 18 and 36 hours incubation at 37°C.



Figures 4.12 (a-e). Chemical structures of (a) N-salicylidene-3-aminomethylpyridine, (b) 1-hydroxy-5-(4-pyridyl-methyl)-iminonaphthalene, (c) 2-(N,N-dibenzylaminopropionyl)-1-naphthol, (d) 3-hydroxybenzaldehyde-1-(carboxymethyl) pyridinium chloride hydrazone and (e) 7-(N,N-diphenylaminomethyl)-8-hydroxyquinoline.

Evaluation of 8-hydroxycoumarin as an alternative to 1-naphthol for the formation of the Indamine complex in solid media.

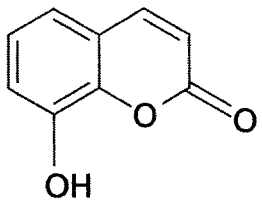


Figure 4.13. Chemical structure of 8-hydroxycoumarin.

The structure of this compound (Figure 4.13) is similar to aesculin and it was felt possible that it may form a chelate with metal ions. Thus, two separate 100 ml volumes of Columbia agar were prepared both containing 67.2 mg of L-alanyl DEPPD, and 40.5 mg of 8-hydroxycoumarin. This produced concentrations of 2.5 mmol l^{-1}. To one volume of medium 25 mg of ferric ammonium citrate was also added. The medium was sterilised and poured as described previously. Plates were then inoculated with the test strains as detailed previously.

Evaluation of L-alanyl-4-4' diaminodiphenylamine as a potential non-spreading substrate for aminopeptidase detection.

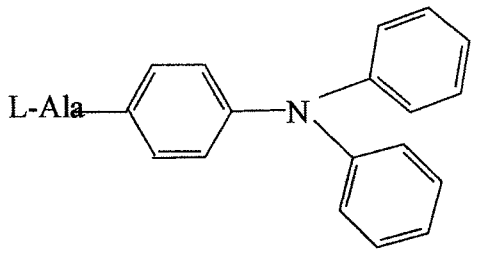


Figure 4.14. Chemical structure of L-alanyl-4-4'diaminodiphenylamine.

The compound L-alanyl-4-4'diaminodiphenylamine (Fig 4.14) may be hydrolysed to produce a coloured complex without the inclusion of a coupling naphthol. For this reason two separate 100 ml volumes of Columbia agar were prepared and 40 mg of L-alanyl-4-4' diaminodiphenylamine was added to each. Finally 10 mg of 3,5-dihydroxy-2-naphthoic acid was added to one of the bottles. The medium was prepared as described previously, inoculated with test NCTC strains and examined after 18 hours incubation at 37°C.

Evaluation of Leucyl-4-amino-2,6-dichlorophenol for detection of L-leucyl aminopeptidase in solid media.

To 100 ml of Columbia agar 20 mg L-leucyl-4-amino-2,6-dichlorophenol and 10 mg of 3,5-dihydroxy-2-naphthoic acid was added. This was prepared

as described previously and inoculated with the test. Production of a coloured reaction product was determined after overnight incubation.

Evaluation of Irgasan (5-chloro-2-(2,4-dichlorophenoxy) phenol) as a substitute for naphthol to restrict the spreading of indamine dye in agar.

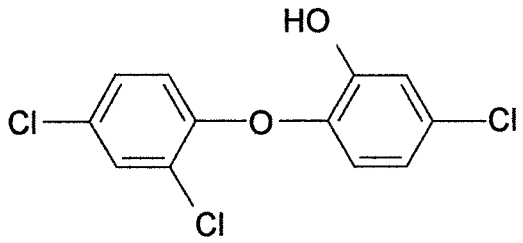


Figure 4.15. Chemical structure of Irgasan.

To 100 ml of Columbia agar containing 67.2 mg L-ala-DEPPD, 72.4 mg of Irgasan (Figure 4.15) was added. Thus both compounds were tested at a concentration of 2.5 mmol l^{-1}. This medium was sterilised, poured and innoculated as described previously. Plates were incubated at 37°C overnight and examined for colour production.

Evaluation of 1,5-naphthalenedisulfonic acid as an alternative to 1-naphthol for the formation of the indamine complex in agar media.

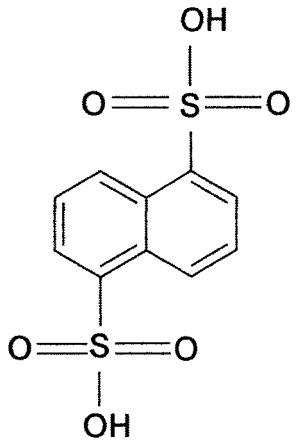


Figure 4.16 Chemical structure of 1,5-naphthalenedisulfonic acid.

A sample of 10 mg of 1,5-naphthalenedisulfonic acid tetrahydrate (Figure 4.16) was dissolved in 1 ml of 0.1 mmol l^{-1} NaOH and added to 100 ml Columbia agar containing 30 mg L-alanyl-DEPPD. The medium was adjusted to pH 7.2 and prepared as described previously. Plates were inoculated and examined after 18 hours incubation at 37°C.

Evaluation of L-alanyl-naphthalene-sulphonyl-diethyl-*p*-phenylenediamine as a potential non-spreading substrate for the detection of aminopeptidase activity.

To 100 ml of Columbia agar 40 mg of L-alanyl-naphthalene-sulphonyl-DEPPD and 10 mg of 3,5-dihydroxy-2-naphthoic acid was added. The medium was sterilised, poured and innoculated as described previously.

In addition a second volume of Columbia agar was prepared containing 40 mg of L-alanyl-naphthalene-sulphonyl-DEPPD and 12 mg N-acetonidophenyl-ethyl-1-hydroxy-2-naphthamide. Plates were prepared and inoculated in identical fashion.

Evaluation of N-(1-naphthyl)ethylenediamine (dihydrochloride) as a substitute for 1-naphthol to restrict the spreading of indamine dye in agar.

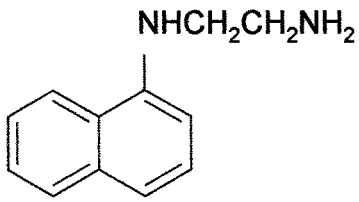


Figure 4.17. Chemical structure of N-(1-naphthyl)ethylenediamine.

To 100 ml of Columbia agar, 30 mg of L-alanyl-DEPPD and 10 mg of N-(1-naphthyl)ethylenediamine dihydrochloride (Figure 4.17) were added. Plates were prepared and inoculated as described previously. Following overnight incubation the plates were examined for colour production.

Evaluation of the diffusion in agar of the indamine dye formed by naphthol and diethyl-*p*-phenylenediamine using a variety of molecules.

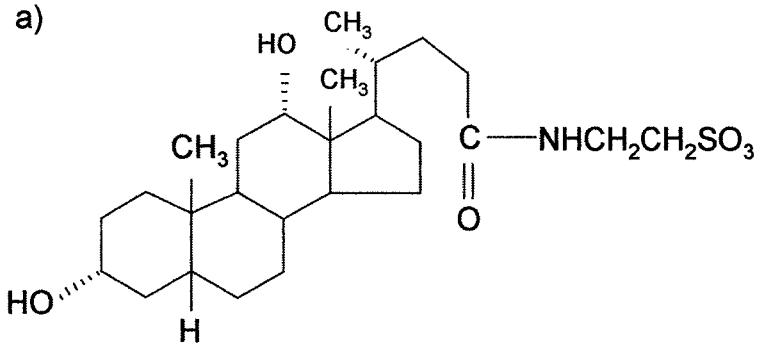
The following compounds were used:

a) Taurodeoxycholic acid (Sodium salt)
b) Taurocholic acid (Sodium salt)
c) Glycodeoxycholic acid (Sodium salt)
d) Deoxycholic acid
e) Dioctyl sulfosuccinate (Sodium salt)
f) 10-Camphorsulfonic acid (Sodium salt)
g) Phytic acid (Calcium salt)
h) Kaolin

Into six separate 100 ml volumes of Columbia agar the following were added, 261 mg of taurodeoxycholic acid, 269 mg of taurocholic acid, 225 mg of glycodeoxycholic acid, 196 mg of deoxycholic acid, 222 mg of dioctyl sulfosuccinate, 127 mg of 10-camphorsulfonic acid, 349 mg of phytic acid (Figures 4.18 a-e), and 5 g of Kaolin. In addition 5.1 mg of 3,5-dihydroxy-2-naphthoic acid, and 67.2 mg of L-ala-DEPPD were added. Thus all compounds were tested at a concentration of 2.5 mmol l^{-1}. The media were prepared, sterilised and poured as described previously. Following a sterility check the plates were inoculated as described in previous methods. In addition, control plates were prepared using the test compound only, and a growth control plate containing Columbia agar only.

The 15 plates were incubated overnight at 37°C and the degree of diffusion of any coloured product recorded.

a)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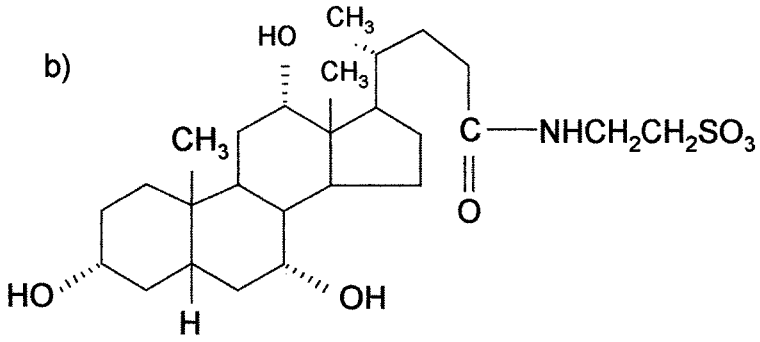
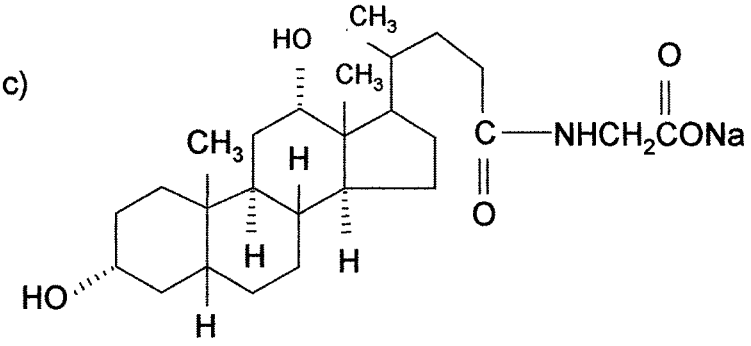
CH3
CH3
CH3
C
NHCH2CH2SO3
O
HO
H

b)


HO
CH3
CH3
CH3
C
NHCH2CH2SO3
O
HO
OH
H

c)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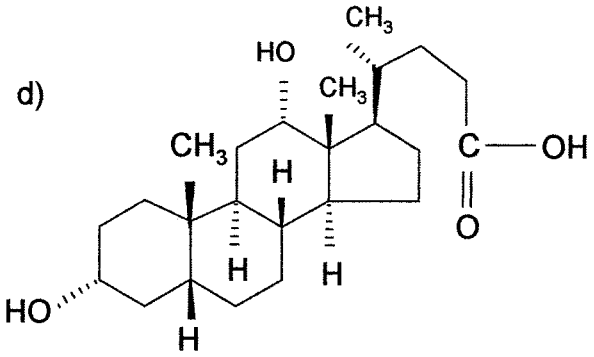
CH3
CH3
O
C
NHCH2CONa
O
CH3
H
H
H
HO
H

d)


HO
CH3
CH3
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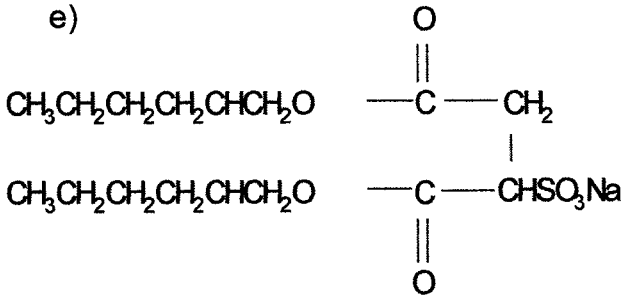
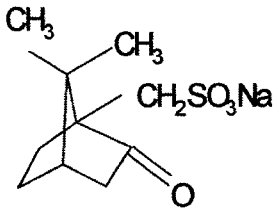
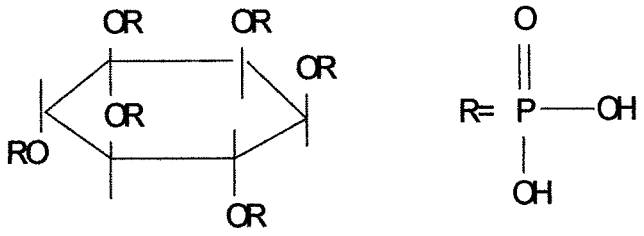
e)



f)



g)



Figures 4.18. Chemical structures of a) Taurodeoxycholic acid, b) Taurocholic acid, c) Glycodeoxycholic acid, d) Deoxycholic acid, e) Dioctyl sulfosuccinate, f) 10-Camphorsulfonic acid, and g) Phytic acid used for limiting spreading of the DEPPD/naphthol complex.

Use of a variety of compounds to limit the diffusion in agar of the coloured complex formed by 3,5-dihydroxy-2-naphthoic acid and DEPPD.

A range of test media were prepared and inoculated with *Klebsiella pneumoniae* (NCTC 10896). Columbia agar was prepared in 100 ml volumes and the following compounds added at both 1 and 10% w/v, Sephadex G-25, DEAE Sephacel, and DEAE Cellulose. Poly (acrylic acid), bovine albumin and Kaolin were added at a concentration of 1% w/v. To all media 5.1 mg of 3,5-dihydroxy-2-naphthoic acid and 67 mg of L-alanyl-DEPPD were then added and the media mixed. Plates were prepared as described in previous methods and NCTC control strains prepared and inoculated as described previously. Growth control plates were prepared using Columbia agar without additives and Columbia agar containing the test compounds only.

Production of a dual substrate system in solid media for the simultaneous detection of two bacterial hydrolyases.

Columbia agar (100 ml) was prepared containing 67 mg of L-ala-DEPPD and 76.6 mg of 1-naphthyl-β-D-galactopyranoside. The medium was sterilised, poured and inoculated with the six NCTC strains as described previously. Plates were examined after overnight incubation for the production of a coloured reaction product.

Due to the interesting findings observed, this experiment was repeated using a wider range of test NCTC strains. Plates were prepared as described previously and organisms inoculated by multi-point. Plates were examined after overnight incubation for the production of a blue coloured product.

Use of L-prolyl-DCAP and 1-naphthyl-β-D-galactoside as a dual substrate system for the presumptive identification of *Serratia sp.*

Columbia agar (100 ml) was prepared containing 67 mg of L-ala-DEPPD and 76.6 mg of 1-naphthyl-β-D-galactopyranoside. The medium was sterilised, and poured as described previously. A total of 140 strains of Enterobacteriaceae, isolated from clinical samples in the Microbiology Department Freeman Hospital, previously identified by API 20E were examined. In addition 10 NCTC *Serratia* control strains were also tested. All strains were prepared and inoculated as described previously. Plates were examined after overnight incubation for the production of a coloured reaction product. In addition all strains were screened in a microtitre format for the presence of β-galactosidase and L-prolyl aminopeptidase activity. This was performed as follows. A double strength stock solution (4 mmol l^{-1}) of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was prepared by dissolving 2.4 g in 20 ml of sterile warm API buffer. A double strength stock solution of L-prolyl-*p*-nitroanilide (5 mmol l^{-1}) was prepared by dissolving 34.8 mg in 20 ml of sterile API buffer. Both solutions were filter

sterilised before use. A 50 µl volume of ONPG and L-prolyl-*p*-nitroanilide was placed into a separate well of a microtitre plate and 50 µl of each organism suspension added. The microtiter plate was then sealed, and placed into a shaker incubator. The wells were examined visibly after overnight incubation at 37°C for the production of a typical yellow reaction product. Control wells contained substrate plus API buffer only.

Results

Determination of the optimal concentration of both L-alanyl-DEPPD and L-alanyl-DCAP in combination with 3,5-dihydroxy-2-naphthoic acid for use in solid media.

The results of these experiments are summarised in Tables 4.1 and 4.2. The optimal concentration, as judged by the concentration of both reactants which produced the most intense reaction product with the least zone of diffusion, was 2.5 mmol l^{-1} for both naphthol and substrate. The medium containing this concentration of L-ala-DEPPD and 3,5-dihydroxy-2-naphthoic acid is shown in Figure 4.19. It was interesting to note that in this experiment *E.faecalis* produced a weakly coloured reaction product indicating the organism possesses only weak L-alanyl aminopeptidase activity, in liquid media however a strong positive reaction was observed. Whilst all Gram-negative strains grew well on the medium both Gram-positive organisms were inhibited on the medium containing both 5 mmol l^{-1} substrate and naphthol. In addition the medium containing L-ala-DCAP was more inhibitory for *S.aureus* as growth was only observed on plates containing a substrate concentration of less than or equal to 1.25 mmol l^{-1}. One of the most interesting observations was that diffusion of the reaction product was more widespread on the medium containing L-ala-DCAP and overall a weaker coloured reaction product was observed as compared to the *p*-phenylenediamine substrate. The greater diffusion would suggest that

either the released substrate core or the coupling dye is more soluble, since the molecular weights of the substrates are similar. This might have been expected, since the presence of a hydroxyl group on the released aminophenol would confer enhanced solubility to the compound.

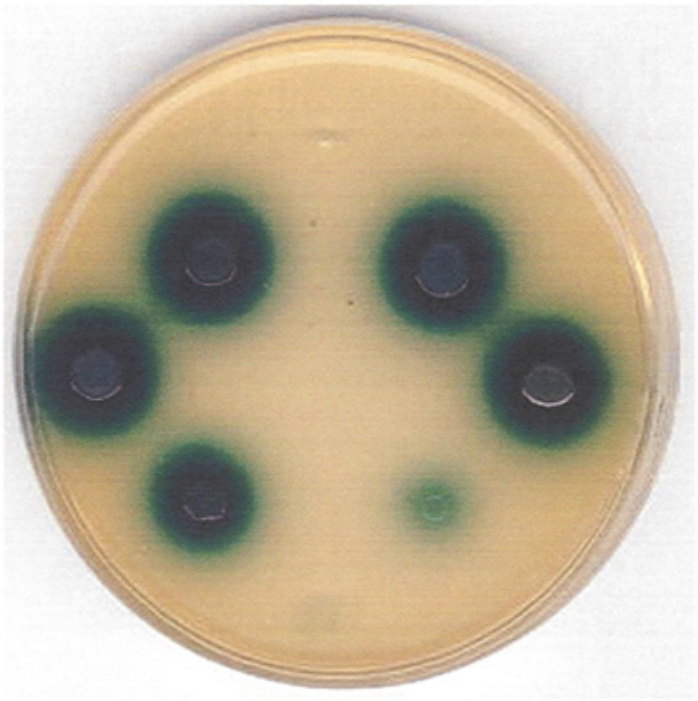


Figure 4.19. Growth of NCTC control strains on a medium containing 2.5 mmol l^{-1} L-ala-DEPPD and 3,5-dihydroxy-2-naphthoic acid. Clockwise from the top are organisms *E.cloacae*, *S.marcescens*, *E.faecalis*, *S.aureus* (no growth), S. *typhimurium*, *K.pneumoniae* and *E.coli*.

Table 4.1 Intensity of coloured reaction product and zone of diffusion shown by a range of Gram-positive and Gram-negaitive organims on Columbia agar containing L-alanyl-DEPPD and 3,5-dihydroxy-2-naphthoic acid.

L-alanyl-DEPPD/3,5-dihydroxy-2-naphthoic acid (mmol l^{-1})	Zone size (mm)	Intensity of reaction product (-/+/++/+++)
5 mmol l^{-1}/5 mmol l^{-1}	20	+++
5 mmol l^{-1}/2.5 mmol l^{-1}	20	+++
5 mmol l^{-1}/1.25 mmol l^{-1}	23	+++
5 mmol l^{-1}/0.625 mmol l^{-1}	25	+++
2.5 mmol l^{-1}/5 mmol l^{-1}	16	+++
2.5 mmol l^{-1}/2.5 mmol l^{-1}	16	+++
2.5 mmol l^{-1}/1.25 mmol l^{-1}	20	+++
2.5 mmol l^{-1}/0.625 mmol l^{-1}	18	++
1.25 mmol l^{-1}/5 mmol l^{-1}	16	++
1.25 mmol l^{-1}/2.5 mmol l^{-1}	15	++
1.25 mmol l^{-1}/1.25 mmol l^{-1}	16	++
1.25 mmol l^{-1}/0.625 mmol l^{-1}	19	+
0.625 mmol l^{-1}/5 mmol l^{-1}	14	+
0.625 mmol l^{-1}/2.5 mmol l^{-1}	14	+
0.625 mmol l^{-1}/1.25 mmol l^{-1}	14	+
0.625 mmol l^{-1}/0.625 mmol l^{-1}	14	+

Table 4.2 Intensity of coloured reaction product and zone of diffusion shown by a range of Gram-positive and Gram-negaitive organims on Columbia agar containing L-alany-4-amino-2,6-dichlorophenol (L-alanyl-DCAP)and 3,5-dihydroxy-2-naphthoic acid.

L-alanyl-DCAP/3,5-dihydroxy-2-naphthoic acid (mmol l^{-1})	Zone size (mm)	Intensity of reaction product (-/+/++/+++)
5 mmol l^{-1}/5 mmol l^{-1}	23	+++
5 mmol l^{-1}/2.5 mmol l^{-1}	23	+++
5 mmol l^{-1}/1.25 mmol l^{-1}	25	+++
5 mmol l^{-1}/0.625 mmol l^{-1}	28	+++
2.5 mmol l^{-1}/5 mmol l^{-1}	23	+++
2.5 mmol l^{-1}/2.5 mmol l^{-1}	22	+++
2.5 mmol l^{-1}/1.25 mmol l^{-1}	22	+++
2.5 mmol l^{-1}/0.625 mmol l^{-1}	26	++
1.25 mmol l^{-1}/5 mmol l^{-1}	19	+
1.25 mmol l^{-1}/2.5 mmol l^{-1}	18	+
1.25 mmol l^{-1}/1.25 mmol l^{-1}	18	+
1.25 mmol l^{-1}/0.625 mmol l^{-1}	19	+
0.625 mmol l^{-1}/5 mmol l^{-1}	16	+
0.625 mmol l^{-1}/2.5 mmol l^{-1}	16	+
0.625 mmol l^{-1}/1.25 mmol l^{-1}	16	+
0.625 mmol l^{-1}/0.625 mmol l^{-1}	15	+

Evaluation of the toxicity of media containing optimal substrate and naphthol concentrations.

The results of the above experiment are summarised in Table 4.3. At a dilution of $<10^{-6}$ individual bacterial colonies could not be counted on any test or control medium. The substrate free control medium showed 61 colonies at a dilution of 10^{-7} for *S.marcescens* and 33 colonies for *E.faecalis*. This equates to the initial suspensions containing 6×10^{10} and 3×10^{10} colony forming units ml^{-1} for *S.marcescens* and *E.faecalis* respectively.

On the medium containing the test substrate L-ala-DEPPD, 18 colonies were observed at a 10^{-6} dilution for *S.marcescens*. This is a growth reduction of 60% compared with the growth control indicating a high degree of toxicity towards the test Gram-negative strain. At a 10^{-6} dilution, 24 colonies were observed for *E.faecalis*, this was a reduction of 28% compared with the growth control at this dilution. This indicates that media containing L-ala-DEPPD is likely to be more toxic towards Gram-negative than Gram-positive species.

On the medium containing L-ala-DCAP *S.marcescens* grew 37 colonies, this is a reduction in bacterial growth of 40% compared with the growth control at this dilution. *E.faecalis* grew 41 CFU's at a dilution of 10^{-6}, a

higher number of CFU's than observed on the control medium. This indicates that the medium is non-toxic towards this organism. This is in contrast to the findings observed in growth experiments detailed in Chapter 2, where very little growth inhibition was observed for any Gram-negative strain tested, except for *K.pneumoniae* which was only partially inhibited by the di-bromo derivative (Figure 2.7.11). The growth of *E.faecalis* was inhibited most noticeably by L-alanyl-4-amino-2,6-dichlorophenol (Appendix 2.3) which is in contrast to the findings in this experiment. The reasons for this cannot be explained. Thus on media containing L-ala-DCAP only the Gram-negative strain was inhibited. The medium containing L-ala-DCAP is less toxic than the medium containing L-ala-DEPPD, whilst this would have little noticeable effect using multi-point inoculated plates, it would be relevant when used in media for the isolation of target organisms. A reduction in bacterial growth of between 40 and 60% would have a serious detrimental effect on the use of DEPPD based substrates in solid media, and the use of DCAP substrates for the isolation of Gram-negative species.

Table 4.3. Growth of *S.marcescens* and *E.faecalis* on media containing L-alanyl-DEPPD, L-alanyl-DCAP and a substrate free control medium. Colonies were counted following overnight incubation (+++ = >100 CFU).

	Control	Control	L-alanyl-DEPPD	L-alanyl-DEPPD	Control	Control	L-alanyl-DCAP	L-alanyl-DCAP
Dilution	*S.marcesens*	*E.faecalis*	*S.marcesens*	*E.faecalis*	*S.marcesens*	*E.faecalis*	*S.marcesens*	*E.faecalis*
10^{-1}	+++	+++	+++	+++	+++	+++	+++	+++
10^{-2}	+++	+++	+++	+++	+++	+++	+++	+++
10^{-3}	+++	+++	+++	+++	+++	+++	+++	+++
10^{-4}	+++	+++	+++	+++	+++	+++	+++	+++
10^{-5}	+++	+++	+++	+++	+++	+++	+++	+++
10^{-6}	61	33	18	24	61	33	37	41
10^{-7}	5	3	2	2	5	3	3	4
10^{-8}	0	0	0	0	0	0	0	0

Evaluation of L-alanyl-4-aminophenol, L-alanyl-DCAP and L-alanyl-DEPPD in the presence of 3,5-dihydroxy-2-naphthoic acid.

As expected from studies in liquid media L-ala-4-aminophenol was hydrolysed to produce a red/orange product (Fig 4.20).

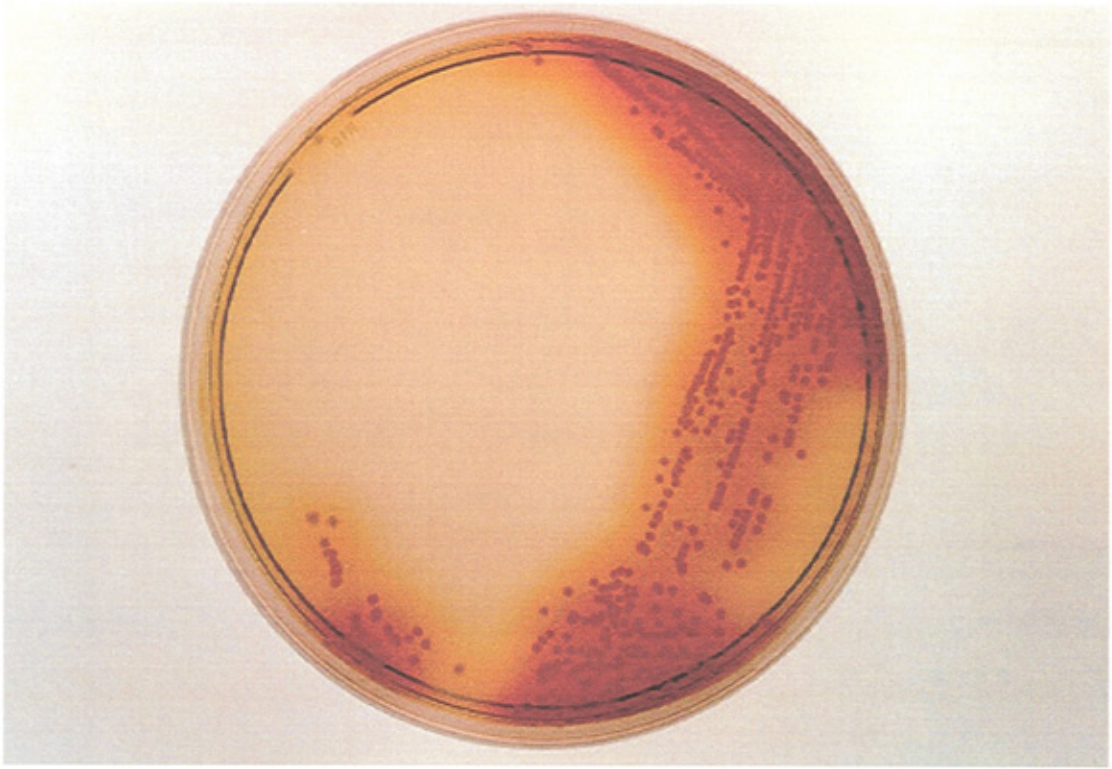


Figure 4.20. Growth and coloured reaction product formed by *E.coli* (NCTC 10418) on a medium containing L-ala-4-aminophenol and 3,5-dihydroxy-2-naphthoic acid.

This diffused throughout the medium with some localisation of colour on the colony mass. This was observed also for media containing L-ala-DCAP (Figure 4.21), which gave a darker pigmentation, and L-ala-DEPPD (Figure 4.22) which gave a blue/green coloured product.

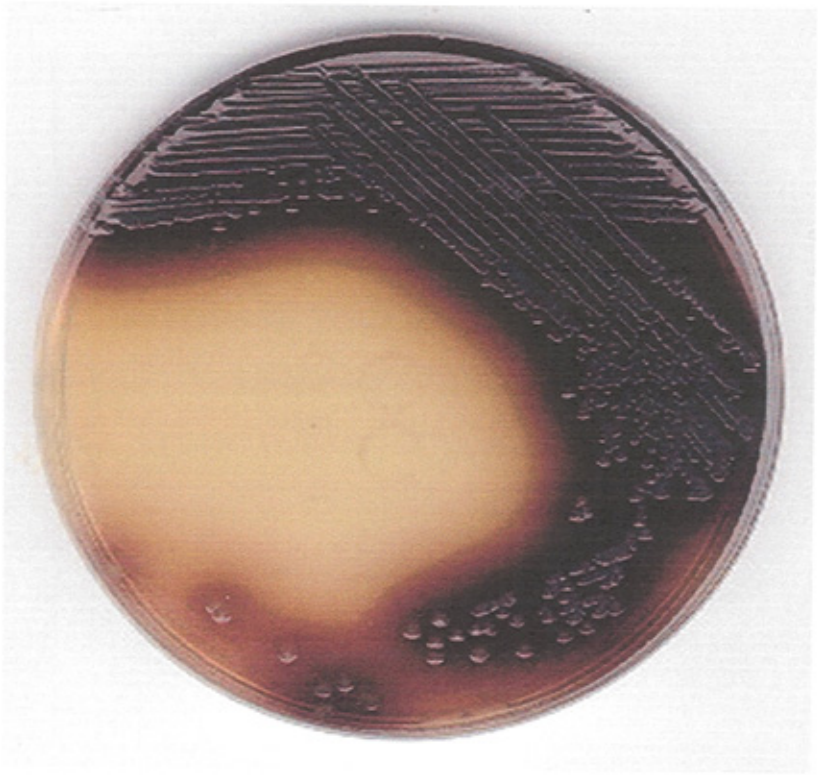


Figure 4.21. Growth of *E.coli* (NCTC 10418) in the presence of L-ala-DCAP and 3,5-dihydroxy-2-naphthoic acid.

Perhaps the most interesting finding was on the medium containing substrate only, to which naphthol was applied following incubation. The naphthol produced a coloured complex, which was similar to that observed when naphthol was incorporated into the medium. This indicated that the released DEPPD "core" diffuses widely through the medium and it is this element of the coupling product that must be modified to produce less diffusion. It would appear from this experiment that modifications to the coupling naphtholic may not produce any benefits with respect to the degree of diffusion around individual bacterial colonies. Alternatively it may be possible to limit the diffusion of the indamine or indophenol dye by the

incorporation of "blocking" agents into the medium, for example, compounds based on bile salts which may interact or bind with the indamine/indophenol complex and limit diffusion. While the diffusion was not total, it was sufficiently widespread to suggest that the detection of target colonies in a heavy mixed culture might be difficult unless some modifications are made.

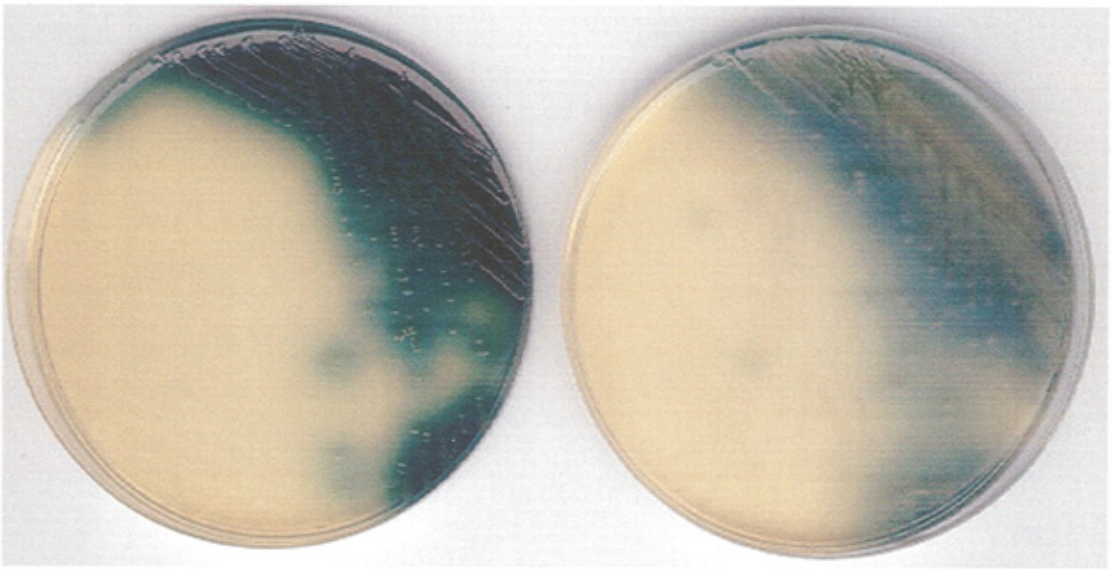


Figure 4.22. Growth of *E.coli* (NCTC 10418) in the presence of L-ala-DEPPD and 3,5-dihydroxy-2-naphthoic acid (left), and L-ala-DEPPD only overlayed with a solution of 3,5-dihydroxy-2-naphthoic acid (left).

Evaluation of the effect of the chelating agent, ferric ammonium citrate on diffusion of the coloured reaction product in solid media containing L-alanyl-DEPPD or L-alanyl-4-amino-2,6-dichlorophenol in the presence of 3,5-dihydroxy-2-naphthoic acid.

In this experiment, both media showed formation of the coloured reaction product, as expected. However there was no detectable difference in either the intensity of the coloured reaction product as judged visibly at any concentration of ferric ammonium citrate used. More importantly there was no difference in the zone size of the diffused reaction product in the presence of any concentration of ferric ions. This indicates that the diffused coloured product fails to form metal chealtes with ferric ions. It is highly unlikely that other metal ions used to form chelates with released chromogens e.g. Mg^{2+} or Al^{3+} would form chelates with these test compounds. Since no differences were observed between test and control plates no photographs were produced, and this line of study was not continued.

Evaluation of L-alanyl substrates based on 4-dimethylaminoaniline (N,N-dimethyl-*p*-phenylenediamine) as potential non-diffusible substrates in solid media.

The substrate, N-(4-alanylamidophenyl)-N-methylaminomethyl-1,3-dioxolane (Figure 4.8a) showed no inhibitory effects when compared to the substrate and naphthol free control plate. For all Gram-negative and Gram-

positive organisms tested no coloured product was observed, even on prolonged incubation for 48 hours. Substrate b, N-(4-alanylamindophenyl)-N-methylaminobutyryl-2-napthalenesulphonylhydrazine (Figure 4.8b) also produced no visible inhibition of bacterial growth. Of the Gram-negative strains tested only *E.coli* and *K.pneumoniae* generated a coloured reaction product. Both produced a weak blue colour which diffused throughout the medium. The Gram-positive strains as expected produced no coloured reaction product, presumably due to negligible production or expression of L-alanine aminopeptidase activity.

Substrate c, 4,4-dialanylamidodiphenylamine (Figure 4.8c) also showed no visible inhibitory effects. No strain produced a coloured reaction product even after prolonged incubation. Substrate d, 2,4-dialanylamido-4-methoxydiphenylamine (Figure 4.8d) completely inhibited the growth of *S.aureus*. No inhibition of any Gram-negative strain, or *E.faecalis* was observed. However, no colour reaction product was evident even on prolonged incubation. Finally, 4-alanylamido-4-diethylaminodiphenylamine (Figure 4.8e) produced no coloured reaction product with any of the strains tested and no inhibitory effects were observed. Due to the poor reactivity of these substrates photographs were not produced, and this aspect of the project was not continued.

Examination of colour produced by L-alanyl derivatives of three substituted derivatives of 4-aminophenol in solid media.

Some inhibitory effect was noted for all test strains grown on media containing L-alanyl-4-amino-2,6-di-isopropyl-phenol and 3,5-dihydroxy-2-naphthoic acid. Of the Gram-negative strains tested only *E.coli* (Figure 4.23), *K.pneumoniae,* and *S.marcescens* produced a coloured reaction product. Orange/red colonies were observed with some colour diffusion into the surrounding media.

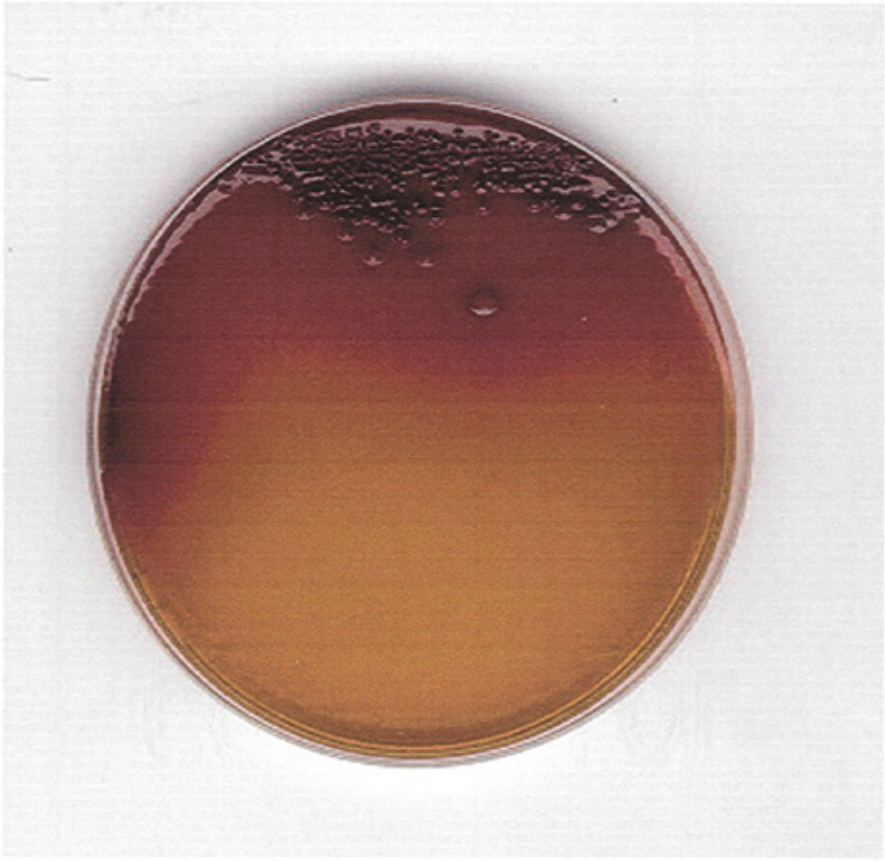


Figure 4.23 Growth of *E.coli* (NCTC 10418) on media containing L-alanyl-4-amino-2,6-di-isopropyl-phenol and 3,5-dihydroxy-2-naphthoic acid.

The substrate L-alanyl-4-amino-2,6-ditertiary butyl-phenol did not appear to inhibit the growth of any test organisms. Similar to the di-isopropyl derivative only *E.coli, K.pneumoniae,* and *S.marcescens* produced a coloured reaction product, which was the same colour and diffused to the same extent as the di-isopropyl derivative. All strains were completely inhibited by L-alanyl-4-amino-2,6-dimethyl-phenol in the presence of 3,5-dihydroxy-2-naphthoic acid. There was no discernible advantage between the use of any of these derivatives and the use of L-ala-4-aminophenol.

Evaluation of three extended L-alanyl-4-aminophenol substrates in solid media.

None of the three substrates visibly inhibited the growth of any of the test Gram-negative or Gram-positive control strains tested. However no coloured reaction product was observed even on prolonged incubation. Due to the non-reactivity of these substrates, photographs were not produced. As a result no further experimental work was performed using these substrates.

Evaluation of L-alanyl-N-phenyl-*p*-phenylenediamine in solid media for the formation of an indamine complex.

Figure 4.24 shows the coloured complex formed by *E.coli* NCTC 10418 in solid media containing this substrate. As can be seen, the pale violet

complex diffused widely throughout the medium. All of the Gram-negative strains tested produced this reaction with no discernible differences between diffusion of the coloured complex, or localisation of the colour on individual bacterial colonies. No inhibition of bacterial growth was evident, as judged visibly. As expected the Gram-positive control strains grew well on the medium but formed no coloured reaction product. The coloured complex formed was less intense than that obtained with L-alanyl-DEPPD and 3,5-dihydroxy-2-naphthoic acid after 18 hours incubation (Figure 4.22). Despite the reactivity of this substrate it shows no significant advantages over L-alanyl-DEPPD and 3,5-dihydroxy-2-naphthoic acid.

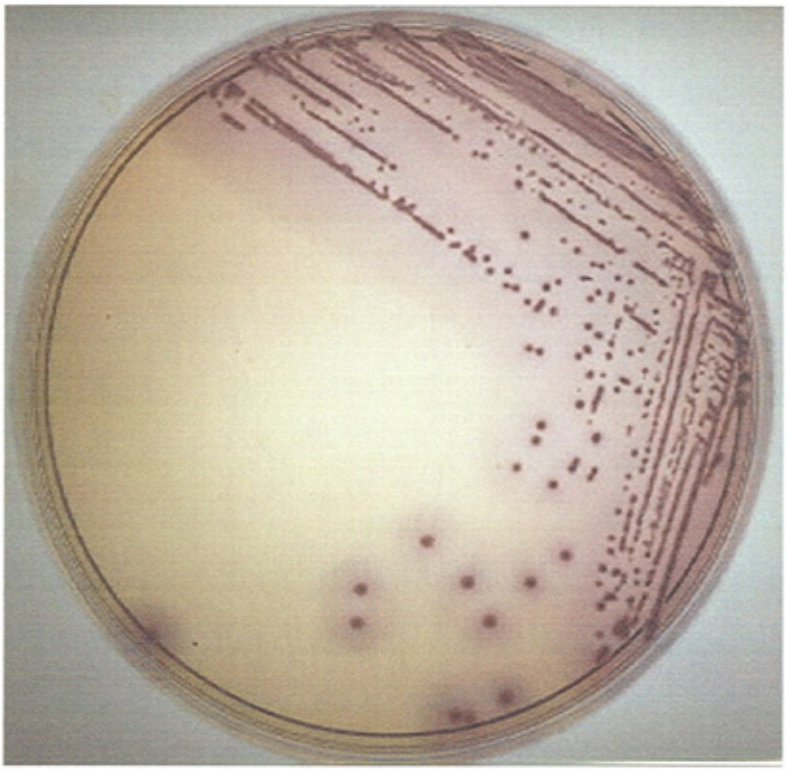


Figure 4.24. Growth of *E.coli* (NCTC 10418) on media containing L-alanyl-N-phenyl-1,4-phenylenediamine and 3,5-dihydroxy-2-naphthoic acid.

Evaluation of a range of compounds as alternatives to naphthol for the formation of coloured complexes with L-alanyl-DEPPD in solid media.

On the medium containing N-salicylidene-3-aminomethylpyridine (Figure 4.12a), all of the Gram-negative strains grew well but produced no coloured reaction product on the medium. This finding may not have been entirely surprising since the side chain may have interfered with the coupling of the released DEPPD core. Additionally while all other strains grew well, growth of *S.aureus* was completely inhibited. The medium containing 1-hydroxy-5-(4-pyridyl-methyl)-iminonaphthalene, (Figure 4.12b) again inhibited the growth of *S.aureus*. All Gram-negative strains grew well on this medium and produced a deep blue colour due to hydrolysis of L-alanyl-DMPPD and subsequent coupling with the extended naphthol derivative. The colour produced diffused readily and was largely analogous with the use of DEPPD/1-naphthol. The extended naphthol, 2-(N,N-dibenzylaminopropionyl)-1-naphthol (Figure 4.12c) failed to generate any coloured reaction product although all test strains grew well on the medium. The hydrazone derivative, 3-hydroxybenzaldehyde-1-(carboxymethyl) pyridinium chloride hydrazone (Figure 4.12d) inhibited the growth of all test organisms as compared to growth control media. As would be expected, no coloured reaction product was observed, with the most likely explanation being the presence of the side chain at position 3 on the phenol ring. This would be expected to strongly interfere with the DEPPD and napthol

coupling at position 4 on the ring. The novel quinoloine derivative, 7-(N,N-diphenylaminomethyl)-8-hydroxyquinoline, (Figure 4.12e) also significantly inhibited the growth of all test organisms. No coloured reaction product was observed for any test strain. Control plates produced the expected dark green coloured complex. Such derivatives have been shown previously to be toxic for several bacterial species (Albert, 1953).

Evaluation of 8-hydroxycoumarin as an alternative to naphthol for the formation of the indamine complex in solid media.

No visible colour was observed after 24 hours incubation in the medium containing 8-hydroxycoumarin and L-alanyl-DEPPD. After 48 hours a faint green colour was observed around the colonies, diffusing into the surrounding medium. The presence of a metal ion, in the form of ferric ammonium citrate, made no difference visibly to diffusion of the coloured complex. This was to be expected since the compound obviously fails to couple readily with released DEPPD. Due to the poor reactivity of this compound photographs were not produced. The results would suggest that 8-hydroxycoumarin has little value as an alternative to naphthol for coupling with released DEPPD.

Evaluation of L-alanyl-4-4' diaminodiphenylamine as a potential non-spreading substrate for aminopeptidase detection.

Figure 4.25 shows that in the absence of a coupling naphthoic a deep blue/violet coloured complex was formed when *E.coli* (NCTC 10418) was grown on a solid medium containing this substrate. As can be observed widespread diffusion of the coloured reaction product through the agar was evident.

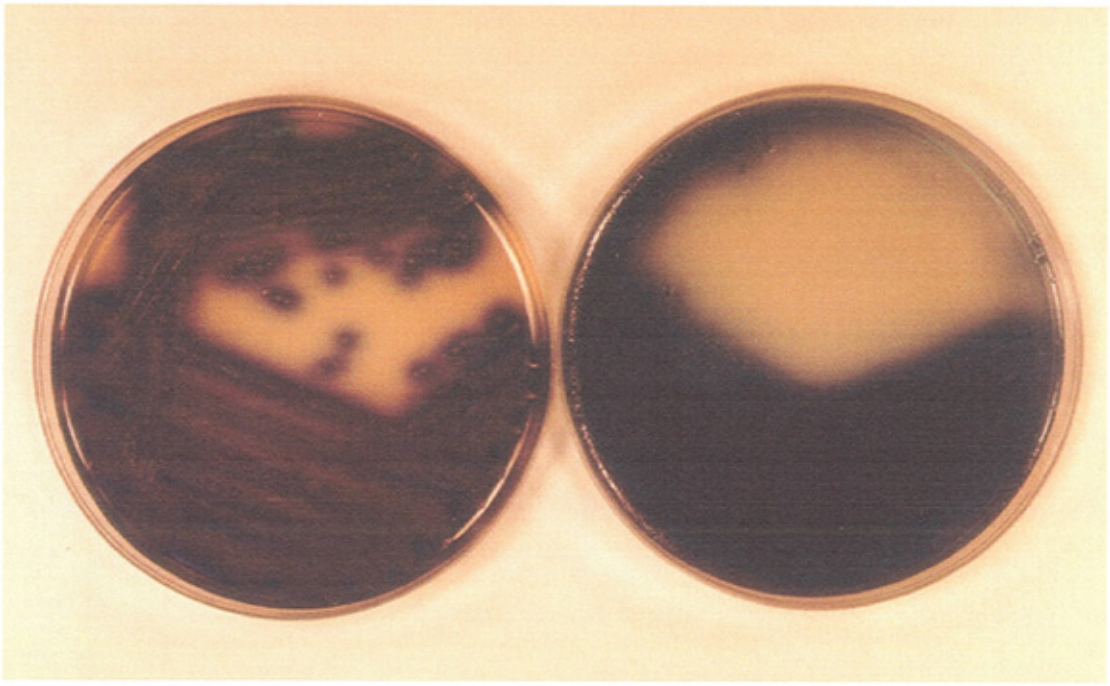


Figure 4.25. Growth of *E.coli* (NCTC 10418) in the presence of L-alanyl-4-4' diaminodiphenylamine (left) and with the test compound plus 3,5-dihydroxy-2-naphthoic acid.

With the inclusion of a coupling naphthoic, the same intense blue/violet colouration was also generated, diffusing widely through the medium.

Despite the intensity of the reaction product, and the lack of a required coupling agent, this substrate would appear to offer little advantage over L-alanyl-DEPPD mainly due to the diffusion of the coloured product.

Evaluation of Leucyl-4-amino-2,6-dichlorophenol for detection of L-leucyl aminopeptidase in both liquid and solid media.

All Gram-negative and Gram-positive strains tested produced a significant reaction with this substrate as observed by the development of a strong purple coloured reaction product. Surprisingly, *S.pyogenes* was reactive, an organism not documented as a producer of L-leucyl aminopeptidase. The reaction product showed moderate diffusion through the agar as expected. Figures 4.26 show the reactivity of this substrate in an agar medium with *E.faecalis*. The reaction was not particularly strong with Gram-positive strains, as observed in Figure 4.26. This would suggest that this substrate would be better suited to use in liquid media since it would be impossible to differentiate the target organism in mixed culture on an agar-based medium. A DEPPD derivative may have yielded a more intense reaction product, which could be more useful in solid media as the coupling product is visibly more intense and would also diffuse to a more limited degree.

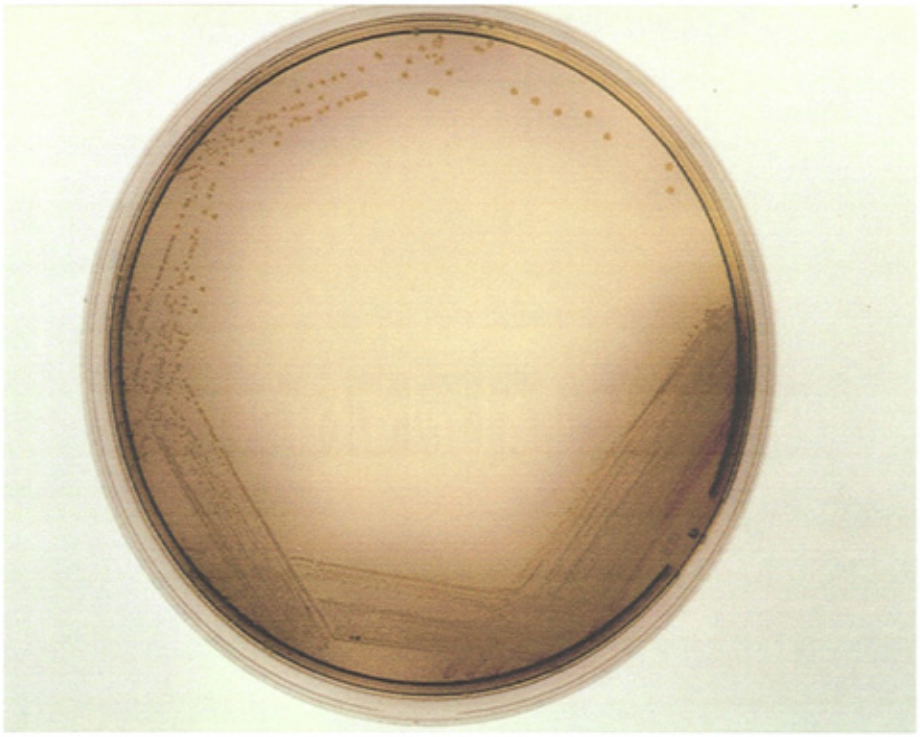


Figure 4.26. Growth of *E.faecalis* (NCTC 755) on a medium containing L-leucyl-4-amino-2,6-dichlorophenol and 3,5-dihydroxy-2-naphthoic acid.

Evaluation of Irgasan (5-chloro-2-(2,4-dichlorophenoxy) phenol) as a substitute for naphthol to restrict the spreading of indamine dye in agar

Two of the organisms under test, *E.coli* and *K.pneumoniae*, did not grow in the presence of Irgasan. The other three organisms produced pale green colonies and it was noticeable that this colouration was more restricted to bacterial colonies than that observed in any previous experiment. After 48 hours incubation the colour of the colonies was significantly deeper but diffusion of the reaction product into the surrounding medium was still evident (Figure 4.27). Despite this high degree of colour localisation, Irgasan is clearly limited in its applicability in general purpose agar media as

it is well-known to exhibit significant inhibitory effects (Hussain *et al.*, 2001) at low concentrations of 4 mg l^{-1}. This was evident by the toxicity towards both *E.coli* and *K.pneumoniae;* however this agent is incorporated into selective media for the isolation of *Yersinia enterocolitica* from stool samples (Jiang *et al.*, 2000). The ability of Irgasan to react with DEPPD and presumably aminophenol may be exploited in the design of media for such organisms that are resistant to Irgasan. Further studies would be required to confirm this finding using strains of *Y.enterocolitica* and other *Yersinia spp.*

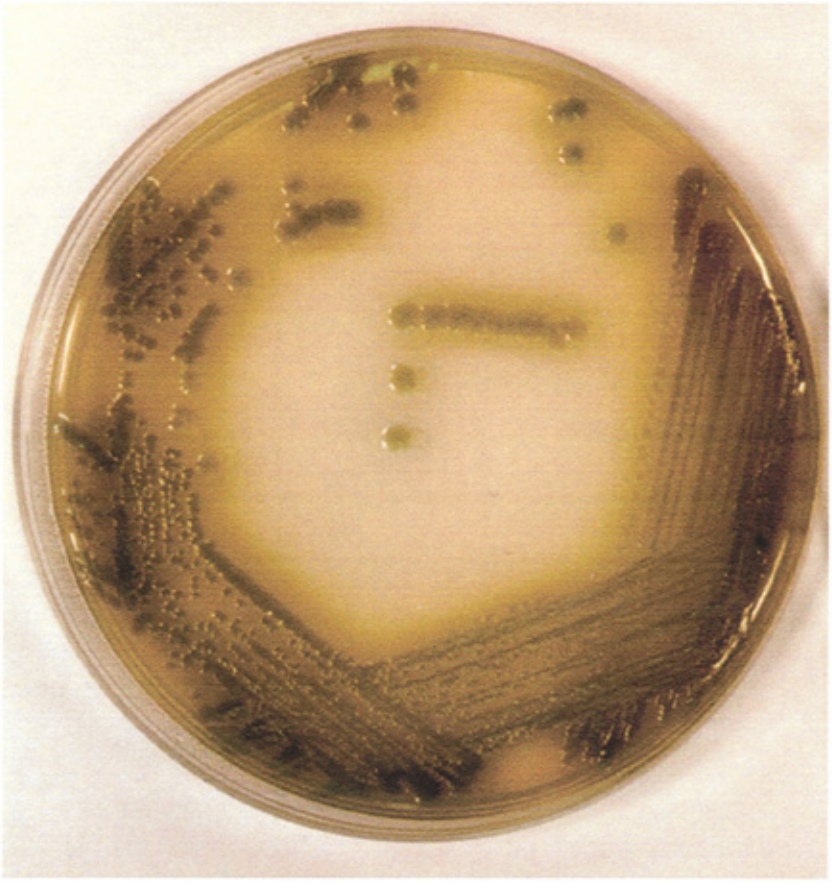


Figure 4.27 Growth of *P.aeruginosa* (NCTC 10662) on Columbia agar containing L-alanyl-DEPPD and Irgasan.

Evaluation of 1,5-naphthalenedisulfonic acid as an alternative to 1-naphthol for the formation of the indamine complex in agar media.

None of the strains tested were inhibited by 1,5-naphthalenedisulfonic acid. Gram-negative strains produced a dark green colouration which diffused widely in Columbia agar, due to the reaction of 1,5-naphthalenedisulfonic acid with DEPPD released by alanyl aminopeptidase activity (Figure 4.28). This compound would appear to offer no advantage over the use of naphthol due to this diffusion.

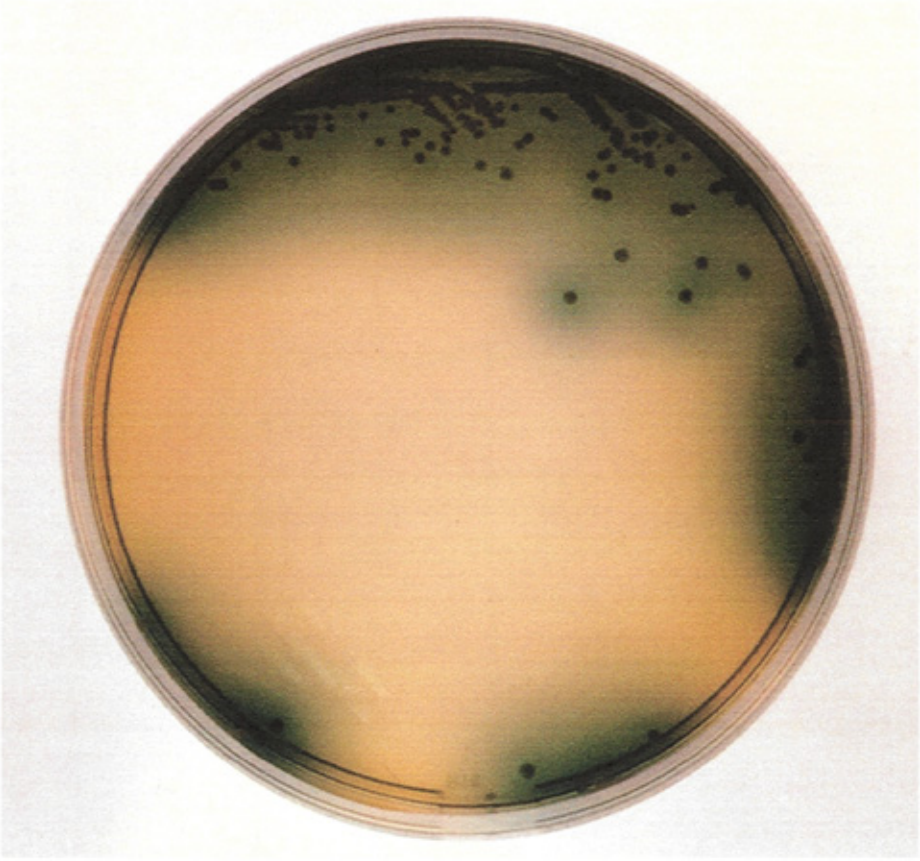


Figure 4.28. Growth of *K.pneumoniae* (NCTC 10896) Columbia agar containing L-alanyl-DEPPD and 1,5-naphthalenedisulfonic acid.

Evaluation of L-alanyl-naphthalene sulphonyl-diethyl-*p*-phenylenediamine as a potential non-spreading substrate for the detection of aminopeptidase activity.

None of the organisms tested showed any visible inhibition by the use of L-alanyl-naphthalene sulphonyl-DEPPD. When tested in the presence of a coupling naphthol, Gram-negative bacteria produced a pale green colouration which readily diffused. In the presence of N-acetonidophenyl-ethyl-1-hydroxy-2-naphthamide (Fig 4.29) the colour produced was significantly less intense and readily diffused through the agar medium

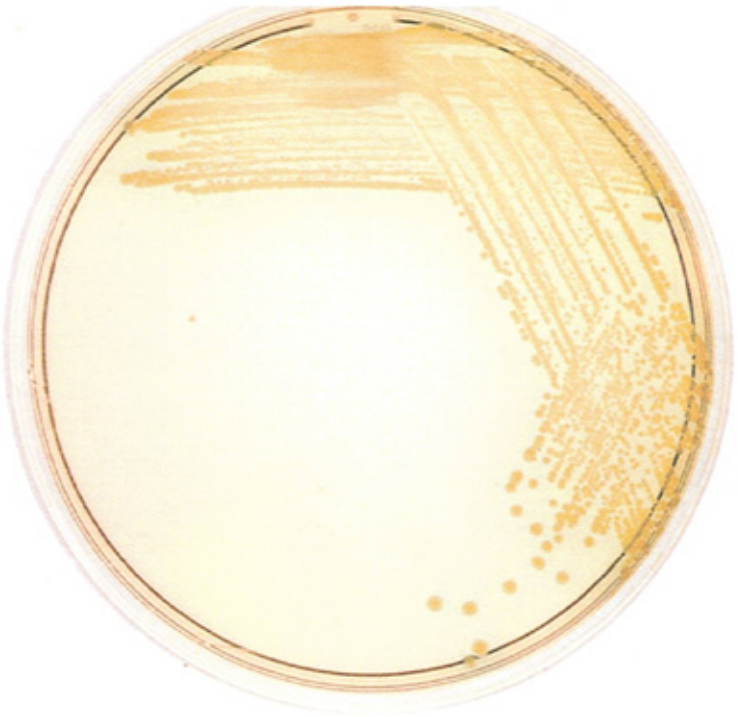


Figure 4.29. Growth of *K.pneumoniae* (NCTC 10896) on Columbia agar containing L-alanyl-naphthalene-sulphonyl-DEPPD and N-acetonidophenyl-ethyl-1-hydroxy-2-naphthamide.

Evaluation of N-(1-naphthyl)ethylenediamine (dihydrochloride) as a substitute for naphthol to restrict the spreading of indamine dye in agar.

N-(1-naphthyl)-ethylenediamine did not inhibit the growth of any of the organisms tested. Gram-negative strains produced a faint green colouration, which diffused through the agar. This compound was therefore regarded as a poor alternative to naphthol for the formation of indamine dye with DEPPD and therefore photographs were not produced, and no further experimental work was carried out.

Evaluation of the diffusion in agar of the indamine dye formed by naphthol and diethyl-*p*-phenylenediamine using a variety of molecules.

None of the compounds tested; taurodeoxycholic acid, taurocholic acid, glycodeoxycholic acid, deoxycholic acid, dioctyl sulfosuccinate, 10-camphorsulfonic acid, phytic acid, or kaolin had a significant effect on the diffusion of the indamine dye produced by the reaction of naphthol and DEPPD. Figure 4.30 show the effects of two of the test compounds on the growth of *Salmonella typhimurium* (NCTC 74) and its effect on diffusion of the coloured complex.

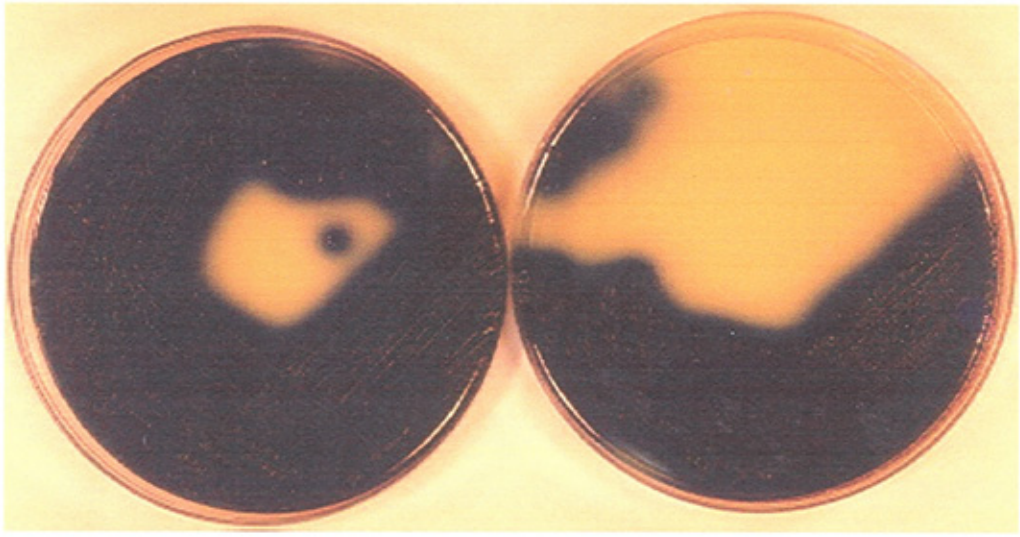


Figure 4.30. The effect of 1 mg ml^{-1} taurodeoxycholic acid (left) and 1 mg ml^{-1} glycodeoxycholic acid (right) on the growth of *S.typhimurium* with L-alanyl-DEPPD and 3,5-dihydroxy-2-naphthoic acid.

Use of a variety of compounds to limit the diffusion in agar of the coloured complex formed by 3,5-dihydroxy-2-naphthoic acid and diethyl-*p*-phenylenediamine.

Sephadex was found to have no influence on colour diffusion at a concentration of 1 %. At 10% however colour diffusion was slightly reduced and the bacterial growth was not inhibited. The addition of DEAE-Sephacel at 1% reduced the observed diffusion slightly but at 10% diffusion of the coloured complex was significantly less. This may have been due to the inhibitory effect of the compound on bacterial growth, causing less substrate to be hydrolysed and producing a smaller amount of coloured reaction product. DEAE-Cellulose produced the same results at 1% and 10% in that the compound proved non-inhibitory. However diffusion of the

coloured complex was only slightly reduced. Poly (acrylic acid) which when used at 1% caused inhibition of bacterial growth, therefore little blue colour was produced. Bovine albumin, which at 1% prevented the agar from setting as a smooth, level medium, and did not stop the diffusion of the coloured reaction product. Finally Kaolin, whilst visibly appearing non-inhibitory, diffusion of the indamine dye into the surrounding medium was evident (Figure 4.31). Further investigations therefore using these compounds was not pursued.

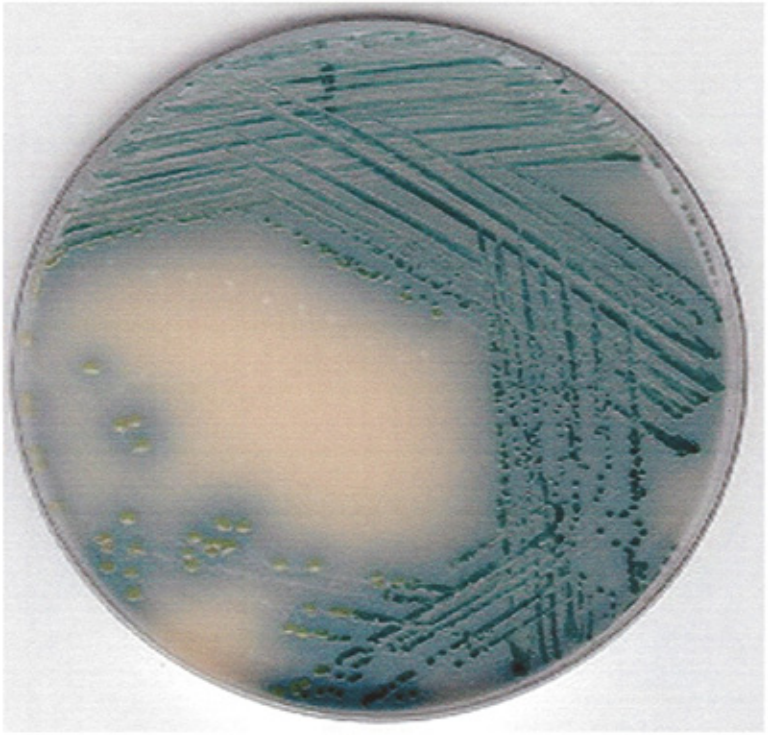


Figure 4.31. Growth and colour produced by *E.coli* (NCTC 10418) on media containing kaolin, L-alanyl-DEPPD and 3,5-dihydroxy-2-naphthoic acid.

Evaluation of a dual substrate system in solid media for the simultaneous detection of two bacterial hydrolyases.

A blue coloured reaction product was observed on media containing L-alanyl-DEPPD and 1-naphthyl-β-D-galactopyranoside with *E.coli*, *E.cloace*, and *K.pneumoniae* (Fig 4.32). No coloured product was observed on the medium containing *S.typhimurium* or *S.aureus*. Since the only test strains which produce both enzymes are *E.coli, E.cloacae, and K.pneumoniae,* this preliminary experiment confirms the validity of a dual substrate system. *Salmonella typhimurium* produces only L-alanyl aminopeptidase and no colour production was evident indicating the absence of β-galactosidase and therefore, no free naphthol released for coupling. The *S.aureus* control strain produces neither of these enzymes and thus no coloured product was evident.

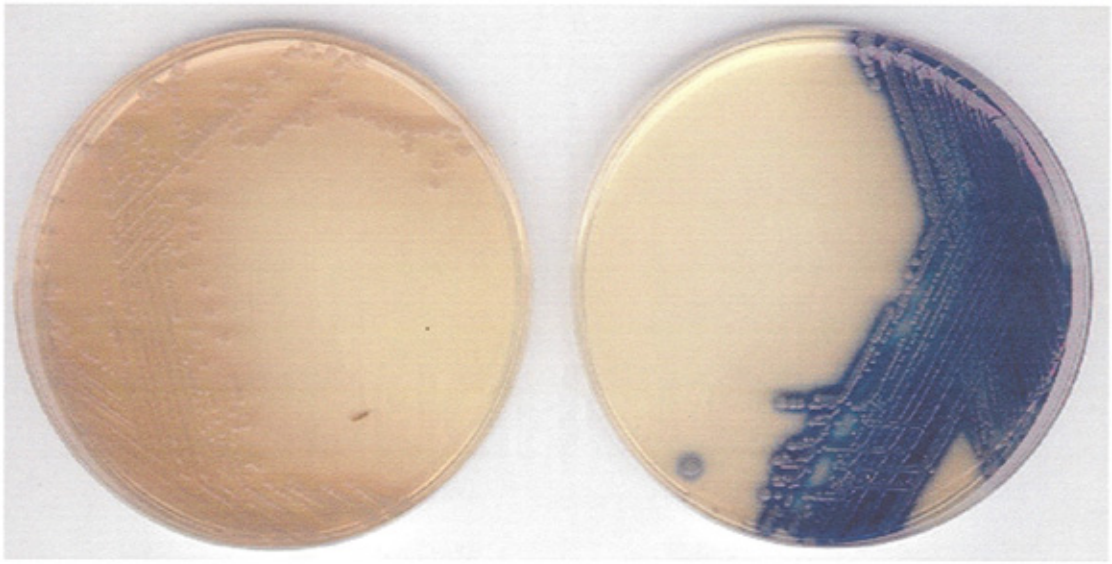


Figure 4.32. Shows growth of *S.typhimurium* (left) and *E.coli* (right) on a medium containing L-alanyl-DEPPD and 1-naphthyl-β-D-galactoside.

Use of L-prolyl-DCAP and 1-naphthyl-β-D-glucoside as a dual substrate system for the presumptive identification of *Serratia spp.*

Table 4.4 shows the full results of this experiment and Figure 4.33 the reaction of a number of test strains.

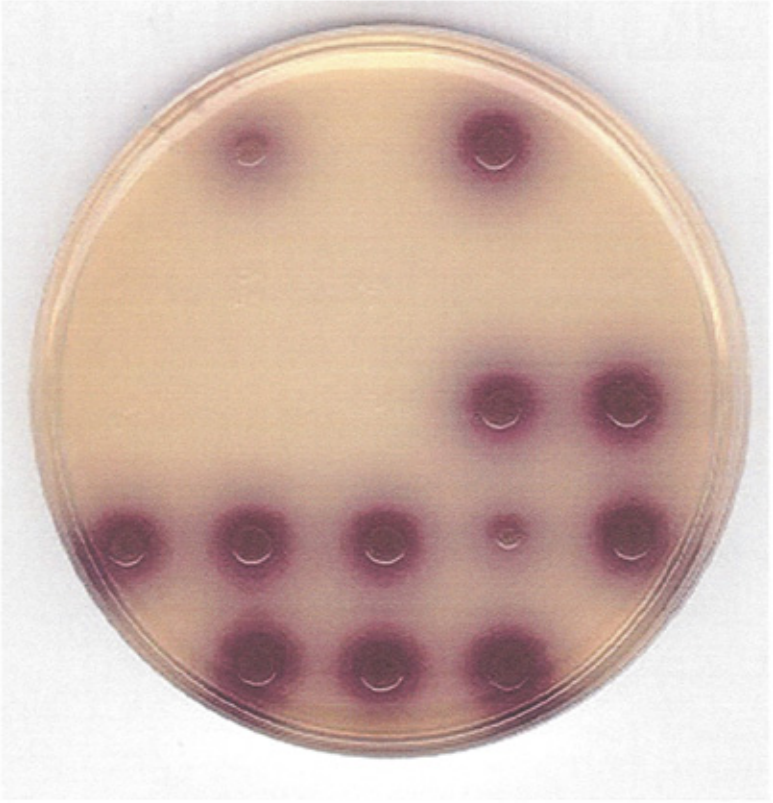


Figure 4.33. In five rows from left to right, reaction of *S.marcescens*, blank control, and *S.liquefaciens.* Row 2, *E.coli*, *K.pneumoniae*, *K.oxytoca*, *E.cloacae*, and *M.morganii.* Row 3, *Salmonella* spp, *C.freundii*, *C.diversus*, and ten NCTC *Serratia* control strains on a medium containing L-prolyl-DCAP and 1-naphthyl-β-D-glucoside.

Table 4.4 shows the results of 150 test strains of Enterobacteriaceae in the presence of three different substrates. As can be seen from the table several test strains of Enterobacteriaceae with the exception of *M.morganii*, *Salmonella spp* and four strains of *H.alvei* were positive for the presence of β-glucosidase using ONPGLU (Table 4.4). The test strains were far less reactive when tested for the presence of L-prolyl-aminopeptidase using L-prolyl-*p*-NA. All 21 wild strains of *Serratia spp* were positive, as were all 10 of the NCTC control strains. Of the wild strains of Enterobacteriaceae tested, 2 strains of both *K.pneumoniae* and *C.freundii* were positive, as was one strain of *H.alvei*. When multipoint inoculated onto the dual substrate medium the results of the screening for β-glucosidase and L-prolyl-aminopeptidase activity was in complete agreement with the results of media containing L-prolyl-DCAP and 1-naphthyl-β-D-glucoside, i.e. strains which were positive in both reactions showed a blue/violet colour on the dual substrate medium. These results showed that the strains reacting on the dual substrate medium were screen positive for the presence of both individual test enzymes. Overall, the incorporation of these two test substrates into a solid medium would be highly specific for the presence of *Serratia* spp. Whilst such a medium would not be 100% specific, due to the false positive results obtained with occasional strains of *K.pneumoniae*, *H.alvei*, and *C.freundii*, other agents could be incorporated into the medium in an effort to make it more specific for the detection of *Serratia* spp. The incorporation of an antibiotic agent e.g. cefuroxime, to which *Serratia* spp.

are naturally resistant (MIC >100 mg l^{-1}) and the other strains most often sensitive (Kucers and Bennett, 1987) would enhance the selectivity for the isolation of *Serratia* spp and could form the basis of further work.

Table 4.4. Results of 150 *Enterobacteriaceae* in the presence of L-prolyl-*p*-nitroanilide (L-pro-pNA), ONPGlu and L-prolyl-DCAP in combination with 1-naphthyl-B-D-glucoside (1-Nap-glu).

Organism	No of strains	ONPGlu	L-prolyl-*p*-Na	L-prolyl-DCAP/1-Nap-glu
		(Number of strains positive)		
E.coli	25	0	0	0
K.pneumoniae	16	16	2	2
K.oxytoca	8	8	0	0
S.liquefaciens	12	12	12	12
S.marcescens	9	9	9	9
E.cloacae	16	16	0	0
M.morganii	10	0	0	0
Salmonella spp.	18	0	0	0
C. freundii	10	10	2	1
C. diversus	5	5	0	0
H.alvei	11	7	1	1
S.liquefaciens (NCTC 10861)	1	1	1	1
S.marcescens (NCTC 9741)	1	1	1	1
S.marcescens (NCTC 9743)	1	1	1	1
S.marcescens (NCTC 11935)	1	1	1	1
S.marcescens (NCTC 10211)	1	1	1	1
S.ficaria (NCTC 12148)	1	1	1	1
S.fonticola (NCTC 12147)	1	1	1	1
S.marinorubra (NCTC 10845)	1	1	1	1
S.odorifera (NCTC 11214)	1	1	1	1
S.plymuthica (NCTC 8015)	1	1	1	1

Discussion

The experiments in this Chapter have demonstrated that a coloured product, formed from the reaction of a novel substrate and a naphthol in the presence of a target organism diffuses widely from the bacterial colony. Figure 4.22 shows that the colour is due to diffusion of the released DEPPD core compound upon enzyme hydrolysis, since flooding the plate with naphthol after overnight incubation reveals the extent of the diffusion. One possible mechanism for this was that whilst the two reactants couple in the presence of atmospheric oxygen, the oxygen concentration in the medium is too low for the optimal concentration required for coupling. The addition of an oxidant e.g. potassium ferricyanide may promote more rapid coupling and hence less diffusion, although the toxicity of this and other oxidising agents may counteract any benefits derived from more limited diffusion. The use of oxidising agents would be an interesting avenue of future research.

Whilst the DEPPD and 4-aminophenol cores are both poorly water-soluble, their small molecular size obviously facilitates travel through a solid agar medium. It would be highly unlikely that the indophenol blue complex, which exists as a resonance hybrid, (Figure 4.34) and is highly water insoluble, would diffuse through agar once formed, even if the coupling naphtholic derivative e.g. 3,5-dihydroxy-2-naphthoic acid, contained more solubilising groups than 1-naphthol.

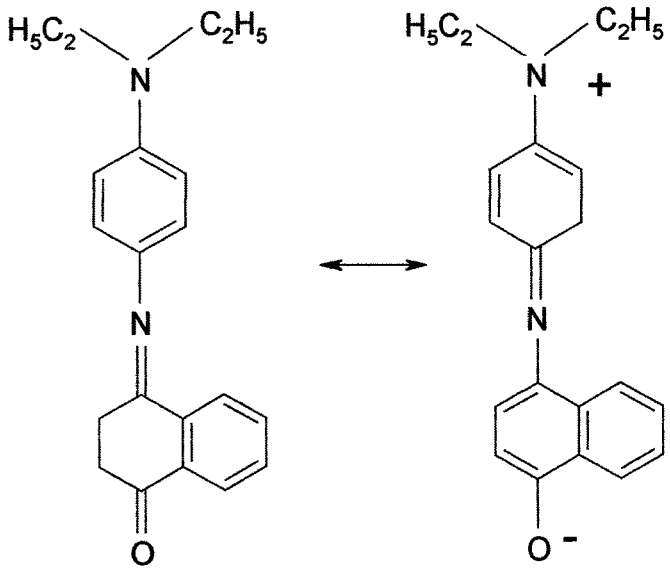


Figure 4.34. Resonance hybrid structure of indophenol blue.

Since the released core compounds appear to diffuse readily through agar, the structure of agar fails to inhibit the diffusion of the released core compounds. Agar consists of a "neutral" fraction of alternating 1,3-linked-β-D-galactopyranose and 2,4-linked, 3,6-anhydro-α-L-galactopyranose moieties (Figure 4.35), and a secondary structure consisting of linear polysaccharide chains (Dean *et al.*, 1985). The integrity of the highly complex structure of agar depends on the secondary structure caused by covalent bonds between the agarose chains (Dean *et al.*, 1985). The pores in the agar are however large enough to be readily penetrated by proteins of high molecular weight.

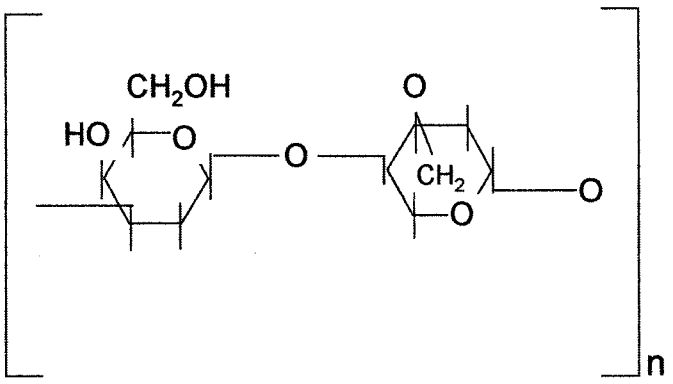


Figure 4.35. Repeating chemical structure of agarose. The hydrogen atoms have been omitted for clarity.

This suggests that the core compounds of low molecular weight generated upon enzyme hydrolysis in this study, would easily penetrate through these agar pores and result in coloured products some distance from the bacterial colonies. Indeed this was observed in initial experiments with all of the L-alanyl substrates tested. A better approach to the production of a substrate would be to either add hydrophobic side chains to the DEPPD or aminophenol core or, alternately, to directly derivatise indophenol blue with an amino acid at the di-methyl/amino position (Figure 4.36). The released compound would be expected to have the chemical properties of indophenol blue, and in particular to be highly water-insoluble. The higher molecular weight of the compound (276) would also be expected to slow diffusion through agar. This approach would also have the advantage in that a

coupling naphtholic would not be required, thus also reducing the potential toxicity of the medium.

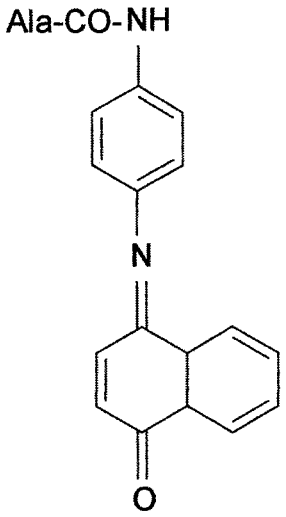


Figure 4.36. Structure of L-alanyl-Indophenol blue.

After preliminary experiments, the work thus shifted to chemical modification of both the enzyme substrate and the coupling naphthol. Initial modifications were made to the substrate, primarily by increasing its hydrophobic nature by the addition of various types of side chains. This approach, however, generated little success. The production of the substrates of L-alanyl-4-amino-2,6-di-isopropyl-phenol, L-alanyl-4-amino-2,6-di-tertiary-butyl-phenol and L-alanyl-4-amino-2,6-dimethyl-phenol (Figures 4.9 a-c) for example, would have been expected to diffuse poorly through agar upon hydrolysis. The tertiary butyl derivative (Figure 4.9b) was expected to be the least soluble, since solubility of alkanes decreases as

the molecular weight increases (Finar, 1975). While coloured compounds were generated, an inhibitory effect was also observed; suggesting the addition of side chains merely produced more toxic derivatives. Kubo e*t al.*, (1995) found a correlation between the alkyl chain length and toxicity in long-chained alcohols and phenols, with most activity observed against Gram-positive bacteria. This would imply that the di-methyl derivative would be the least toxic, although this was not observed in this study. It would seem from these experiments that the shorter the side chain the more toxic the compound is to both Gram-negative and Gram-positive strains. Overall attempts to limit the diffusion of the released core molecule by increasing the hydrophobic nature of the *p*-phenylenediamine or aminophenol, only produced compounds that inhibited bacterial growth and were thus of little further use. The addition of side chains to the 4-aminophenol nucleus must be at the 2 or 6 position on the ring otherwise steric effects would have an effect on enzymic hydrolysis of the amino acid. The addition of side chains to the core compound would probably be better if only an ortho-substituent is added, and the chain length is increased. A better future approach may be to derivatise the core compound with a methoxy or ethoxy group (Figure 4.37). Such groups are angular at the oxygen atom and would be expected to "bend" out of the way giving easier access for the enzyme to its target site. Even a double substitution at the 2 and 6 positions would be expected to allow enzyme hydrolysis. In addition, the inclusion of solubilising groups such as carboxyl, hydroxyl, or sulphonic

acids could make the core compound less toxic. Chemically however, such compounds would be difficult to synthesise.

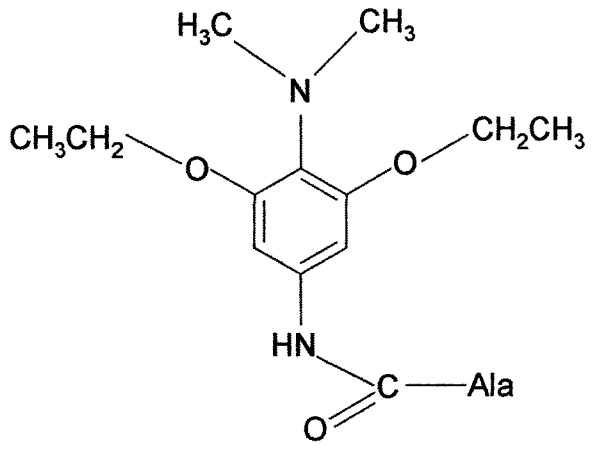


Figure 4.37. Chemical structure of a proposed 2,6-di-methoxy derivative of L-alanyl-DMPPD.

Synthetically easier would be derivitisation of the N,N di-alkyl group with either a single or double-extended side chain. Such a compound, depending on its chemical composition, may diffuse poorly, particularly if the side chains were hydrophobic and the substrate was of such high molecular weight that it traveled less easily through the porous structure of agar. An alternative avenue for study may be in the production of this type of substrate.

Similarly attempts to derivitise the coupling naphthoic molecules (Figure 4.38) either produced coloured reaction products, which were not as intense

as those observed in the coupling between an aminophenol or *p*-phenylenediamine substrate, or again were highly toxic to the bacterial species tested.

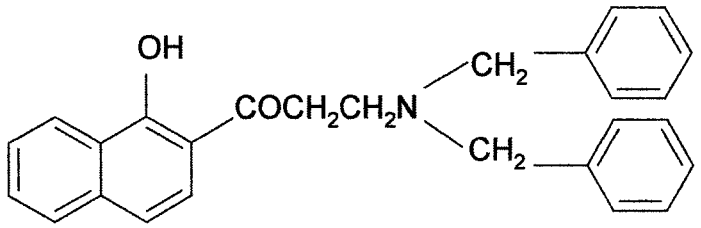


Figure 4.38. Chemical structure of an extended naphthol derivative.

1-naphthol has been shown to exhibit toxicity towards mammalian cells and derivatives have been examined for cytotoxicity and antimicrobial activity by Shen *et al.*, (1995). Their results were in agreement with the experiments described in Chapter 2. Derivatives, especially the 2-hydroxy-methyl-1-naphthol diacetate derivative (Figure 4.39) were shown to be more inhibitory than both ampicillin and kanamycin against a range of organisms.

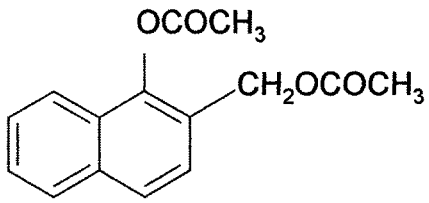


Figure 4.39. Chemical structure of 2-hydroxy-methyl-1-naphthol diacetate (TAC). (Shen *et al.*, 1995).

It was suggested that because of the hydrophobic side chains, the passage of TAC through the high lipid cell envelope of Gram-negative bacteria would account for its potent activity. Most of the extended chain naphthol derivatives examined in this chapter have strongly hydrophobic side chains and thus would be expected to have a high potency towards Gram-negative bacteria.

The incorporation of compounds such as taurocholic acid and camphorsulphonic acid were incorporated to potentially trap the released DEPPD or 4-aminophenol core molecule. Taurocholic acid and similar compounds are bile salts and are negatively charged at most pH values above 4. Bile salts are used in commercially-prepared media to trap the red form of neutral red, which is produced by protonation as the pH of the medium falls due to fermentation of a carbohydrate. This approach is used to detect *Salmonella spp.* from stool samples (Fricker, 1987) on Deoxycholate citrate agar (DCA) and *E.coli* on MacConkey agar (Trepata and Edburg, 1984). Phytic acid (Inositol polyphosphate) has anionic phosphate groups (Barrientos and Murthy, 1996), which might have reacted with the released DEPPD, and the diethyl amino positions, which are positively charged. This would have resulted in a larger complex, which might have resulted in the colour remaining more localised. Other compounds were examined e.g. poly acrylic acid has a cross- linked structure and some ionisable groups, particularly numerous COO^- side

chains. These may have interacted with the positively charged diethyl-amino group on the released core. However none of these produced any significant inhibition of the diffusion of the coloured complex through the medium, showing such effects were not able to reduce the motility of the coloured complexes.

The use of ion chelating resins also failed to produce any reduction in diffusion of the reaction product. DEAE polymers have strongly basic tertiary amine groups attached to the body of the matrix e.g.

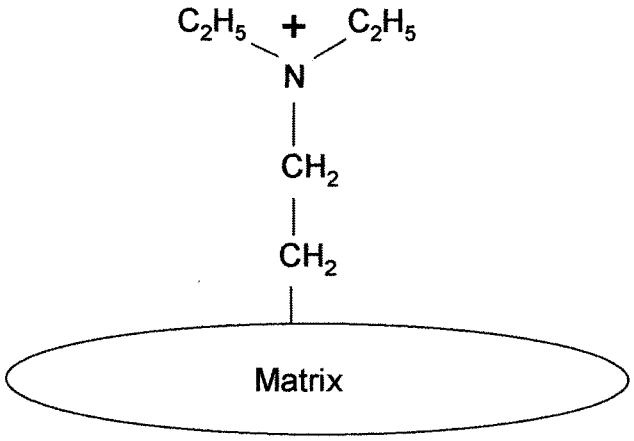


Figure 4.40 Chemical structure of a DEAE polymer showing positively-charged di-ethyl amino ethyl group.

Since the structure is positively charged it would not be expected to bind to either DEPPD or 4-aminophenol or indeed to the indophenol dye (Figure 4.34). The most likely binding would occur with the dye formed upon

coupling of the released substrate core with the dye formed from using 3,5-dihydroxy-2-naphthoic acid as the coupling agent, as shown in Figure 4.41.

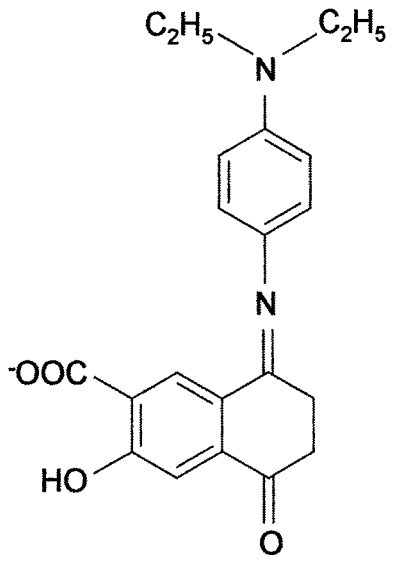


Figure 4.41. Chemical structure of dye formed on coupling between DEPPD and 3,5-dihydroxy-2-naphthoic acid showing negatively charged COO^- group.

Other compounds e.g. bovine serum albumin also failed to limit diffusion of the coloured complex. Albumin in its many forms is known to bind many drugs in a competitive manner (Bhattacharya *et al.*, 2000). This property was investigated in this study but failed to limit diffusion of the coloured complex.

Perhaps the most interesting results were those obtained when a dual substrate system was evaluated. It was clear from the initial experiment using L-alanyl-DEPPD and 1-naphthyl-β-D-galactoside that a dual substrate system would have substantial potential in diagnostic microbiology. The combination of enzymes required for the generation of a coloured product allows targeting of only Gram-negative strains which also produce β-galactosidase. Gram-positive strains which are β-galactosidase-producing will fail to generate a colour by virtue of the lack of L-alanyl-aminopeptidase activity, although in liquid media some strains produced this enzyme. There are several diagnostic possibilities of this system. For example a combination of L-pyroglutamyl-DEPPD and 1-naphthyl-β-D-glucoside would be highly specific for the detection of *Enterococcus* spp. These organisms are known to cause several human infections (Klare *et al.*, 2001) and one of the diagnostic markers used as evidence of faecal pollution of water supplies (Adcock and Saint, 2001). Alternatively a combination of L-prolyl-DEPPD and 1-naphthyl-β-D-glucoside would be highly specific for *Serratia* spp. These organisms are often multi-drug resistant and have been shown to cause outbreaks of disease in several institutions (Villari *et al.*, 2001). Further work in this area would be useful to evaluate these possibilities.

CHAPTER FIVE

Discussion and future proposals

The experiments in this thesis, have clearly demonstrated that coupling occurred between *p*-phenylenediamines, and 1-napthol, with the production of a coloured reaction product, thus validating the "Nadi reaction". Additionally, coupling also occurred between 4-aminophenol and two halogenated derivatives and 1-naphthol. The formation of a red orange product was observed with 4-aminophenol, and a deep blue colour with the 2,6-dichloro derivative. From these initial coupling experiments it was clear that aminophenols could also be used, in addition to *p*-phenylenediamines, for the production of novel chromogenic substrates, as strong coloured reaction products were observed with 1-naphthol. Compounds structurally similar to aminophenols and *p*-phenylenediamines also produced strongly coloured reaction products not only with 1-naphthol but a range of naphthol derivatives. From these coupling experiements a myriad of coloured products were obtained, the major limiting potential being the ease of derivatisation into substrates, and toxicity towards microorganisms. Experiments subsequently demonstrated that some of the "core" compounds and naphthols investigated whilst producing intensly coloured reaction products, were highly toxic to bacterial strains and as such may have produced substrates that would have limited usefulness for the detection of bacteria, particularly in solid media. This was particularly true of 4-chloro-1-naphthol and anthranol.

It was found that the colour and intensity of the reaction products depend upon the ability of the naphthol to couple and form a stable coloured complex. The colour was best demonstrated at or around neutral pH, a finding in agreement with Clark (1972). In addition it was found that substitution of halogen atoms into the indophenol nucleus reduced pK_a so that these derivatives retained a strong blue colour even in acidic conditions. It was evident from the experiments of the present study that the halogenated compounds of 4-aminophenol e.g. 4-amino-2,6-dichlorophenol produced intensely coloured reaction products. The fact that the colour development is optimal at neutral pH lends an advantage over chromogens such as *o*- and *p*-nitrophenols, where the yellow colour development is optimal at an alkaline pH (Bascomb, 1987). Moreover the intensity of the colour produced by coupling of *p*-phenylenediamines and aminophenols is more intense than that generated by either *p*-NA or *o*-NP.

The lack of toxicity of 4-aminophenol and derivatives was surprising, since phenol and especially the chlorinated forms are simple aromatics and would be expected to penetrate bacterial cells easily. Many phenolic compounds are potent bactericides (McDonnell and Russell, 1999) and at relatively low concentrations lyse growing cultures of *E.coli*, Staphylococci and Streptococci (Pulvertaft and Lumb, 1948) inducing a progressive leakage of intracellular components (Lambert and Hammond, 1973). Chlorinated phenolics e.g. 2,4,6-trichlorophenol (Fig 22) are also potent bactericides and

are similar in their membrane damaging effects (Hugo and Bloomfield, 1971).

The toxicity of naphthols was as expected, since naphthol and the 4-chloro derivative were highly toxic. Again the simple aromatic structure would facilitate cell penetration (Rauckman *et al.*, 1989) and promote intracellular damage. Similarly the lack of toxicity of 3,5-dihydroxy-2-naphthoic acid may in part by be explained by the presence of both a carboxyl acid group and a hydroxyl group, which have been demonstrated to significantly reduce antimicrobial activity when compared to the parent compound (Kayser and Kolodziej, 1999). The compound 5[[(1-hydroxy-2-naphthyl)-carbonyl]octadecylamino]-isophthalic acid with its extended side chains was relatively non-toxic towards the Gram-negative strains tested (Table 2). The bulky side chain structure of this compound would have difficulty in penetrating bacterial cell walls and this could account for its lack of toxicity compared to the simple aromatic naphthols (Kayser and Kolodziej, 1999).

The toxicity of L-alanyl substrates was similar to that of the core compounds. *S.aureus* was inhibited significantly by three of the L-alanyl substrates tested. The exceptions were L-alanyl-4-aminophenol and L-alanyl-DMPPD, which were relatively non-toxic. The coupling of an amino acid with a core compound would be expected to have some effect on cell toxicity, although since Gram-positive organisms would fail to

hydrolyse the substrate, the toxicity towards *S.aureus* must be entirely related to the substrate itself rather than to any product. Alternately it is possible that impurities generated in substrate synthesis may account for some toxic effects. It would be envisaged that toxicity towards Gram-negative cells would reflect that observed with core compounds (products of enzyme hydrolysis) since production of the relevant aminopeptidase would release the core compound, and indeed this was observed.

The studies on enzyme kinetics of β-galactoside derivatives of these substrates, showed the enzyme has a similar affinity for both 4-aminophenol substrates as judged by K_m, and catalyses the reaction faster that ONPG as measured by V_{max}. These substrates have been shown to perform well in liquid media compared to commercially available chromogenic substrates, particularly the chlorinated derivative of 4-aminophenol. This compound produced a range of substrates for the detection of glycosidases, with the intensely blue reaction product being more easily observed than that which occurred with nitrophenolics. Indeed a range of derivatives of 4-amino-2,6-dichlorophenol have formed the basis of the API Biomerieux Gold Card system which is an automated method for the identification of Gram-negative microorganisms. Whilst enzyme kinetics were performed using β-galactosidase, and interesting future avenue of research would be for the determination of K_m and V_{max} for a wider range of enzymes, e.g β-glucuronidase, β-glucosidase, and L-alanyl-aminopeptidase. This would

confirm if some of the problems observed with the substrates was due to problems with enzyme affinity, or difficulties in catalytic activity, binding, or release from the active site.

In solid media the novel substrates tested produced a range of typical coloured reaction products. Diffusion into the surrounding medium was a problem, and most of the focus of Chapter 4 was in the analysis of a range of derivatised substrates and coupling agents to overcome this problem. This however resulted in substrates which were highly toxic or failed to remain more localised on the colony mass. This would suggest a limited role in solid media for these substrates although the intensity of the coloured product would warrant further attempts to produce substrates which remain more localised on the colony mass. Possibilities exist to examine synthesised derivatives of indophenol blue as potential non-diffusible substrates. It is still an ambition to produce a derivative of this compound for experimentation in solid media.

The most productive area of research in this thesis was the development of a dual substrate system, a method of identification previously not demonstrated. Using these substrates it was theoretically possible to produce a system which required the production of two distinct enzymes before a reaction product was produced. This was demonstrated using a combination of L-prolyl-4-amino-2,6-dichlorophenol and 1-naphthyl-β-D-glucoside, a

system which produces a reaction product only when both β-glucosidase and L-prolyl aminopeptidase are produced and expressed in a target organism. This was demonstrated using *Serratia sp*, organisms, often multidrug resistant, which causes serious nosocomial infections (Steppberger *et al.*, 2002). Based on the project findings the dual substrate system could be applied to a number of target pathogens. For example the combination of a substrate for β-glucosidase activity and L-pyroglutamy-aminopeptidase would be highy specific for enterococci, and solid media containing appropriate antimicrobials would be useful for the targeting of important nosocomial pathogens during outbreaks. The above combination of substrate plus Vancomycin would be useful for the production of media for the selective isolation of VRE. Such dual substrate systems would also be useful for the specific isolation and identification of facultative Gram-negative organisms and anaerobes. A specific isolation medium could be produced for the detection of *Clostridium perfringens*, an important Gram-positive anaerobe, by the incorporation of substrates for the detection of both phospatase and L-prolyl-aminopeptidase activity. In addition the enteric pathogen *Yersinia enterocolitica* is often difficult to isolate and identify on currently available commercial agar, due to other strains of enterobacteriaceae which grow on CIN and ferment mannitol. Potentially the incorporation of substrates for both α and β-glucosidase activity would produce a clinically useful, highly specific medium for the isolation of this important human pathogen. Incorporation of specific antimicrobials would

further enhance the specificity of any novel medium based on a dual substrate system. In summary the potential applications of a dual substrate system are enormous, with numerous possible substrate permutations for the detection of important human pathogens.

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Appendices

Appendix 2.1: Absorbance increases (405 nm) produced by various organisms in the presence of 4-aminophenols and *p*-phenylenediamine derivatives.

	Time (mins)										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
E.coli/5 mmol l^{-1} 4-aminophenol	0	0.05	0.12	0.18	0.22	0.27	0.31	0.35	0.41	0.45	0.48
E.coli/2.5 mmol l^{-1} 4-aminophenol	0	0.01	0.03	0.04	0.07	0.1	0.12	0.15	0.17	0.19	0.22
E.coli/1.25 mmol l^{-1} 4-aminophenol	0	0	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.04	0.05
E.coli/0.625 mmol l^{-1} 4-aminophenol	0	-0	-0	0	0	0	0.01	0.01	0.01	0.01	0.01
E.coli/0.313 mmol l^{-1} 4-aminophenol	0	-0	-0	-0	-0	-0	-0	0	0	0	0.01
E.coli/0.156 mmol l^{-1} 4-aminophenol	0	-0	-0	-0	-0	-0	-0	-0	0	0	0.02
E.coli/0.078 mmol l^{-1} 4-aminophenol	0	-0	-0	-0	-0	-0	-0	-0	0	0.01	0.03
E.coli/BHI control	0	-0	-0	-0	-0	-0	-0	0	0	0.01	0.1
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
K. pneumoniae/5 mmol l^{-1} 4-aminophenol	0	0.04	0.11	0.17	0.22	0.26	0.31	0.36	0.41	0.46	0.53
K. pneumoniae/2.5 mmol l^{-1} 4-aminophenol	0	0.01	0.03	0.05	0.08	0.11	0.13	0.16	0.18	0.2	0.24
K. pneumoniae/1.25 mmol l^{-1} 4-aminophenol	0	0	0.01	0.01	0.02	0.02	0.03	0.09	0.09	0.1	0.11
K. pneumoniae/0.625 mmol l^{-1} 4-aminophenol	0	-0	0	0	0	0	0.01	0.01	0.01	0.01	0.02
K. pneumoniae/0.313 mmol l^{-1} 4-aminophenol	0	-0	-0	-0	-0	0	0	0	0	0	0.01
K. pneumoniae/0.156 mmol l^{-1} 4-aminophenol	0	-0	-0	-0	-0	-0	-0	-0	0	0	0.02
K. pneumoniae/0.078 mmol l^{-1} 4-aminophenol	*0*	*-0*	*-0*	*-0*	*-0*	*-0*	*-0*	*-0*	*0*	*0*	*0.02*
K. pneumoniae/BHI control	0	-0	-0	-0	-0	-0	-0	-0	0	0	0.1
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
E. cloacae/5 mmol l^{-1} 4-aminophenol	0	0.04	0.11	0.17	0.22	0.26	0.3	0.35	0.41	0.45	0.52
E. cloacae/2.5 mmol l^{-1} 4-aminophenol	0	0.01	0.03	0.05	0.07	0.1	0.13	0.15	0.18	0.2	0.23
E. cloacae/1.25 mmol l^{-1} 4-aminophenol	0	0	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.04	0.05
E. cloacae/0.625 mmol l^{-1} 4-aminophenol	0	-0	0	0	0	0	0.01	0.01	0.01	0.02	0.02
E. cloacae/0.313 mmol l^{-1} 4-aminophenol	0	-0	-0	-0	-0	-0	-0	0	0	0.01	0.03
E. cloacae/0.156 mmol l^{-1} 4-aminophenol	0	0	-0	-0	-0	-0	-0	-0	0	0.01	0.03
E. cloacae/0.078 mmol l^{-1} 4-aminophenol	0	0	-0	-0	-0	-0	-0	-0	0	0.01	0.04
E. cloacae/BHI control	0	-0	-0	-0	-0	-0	-0	-0	0	0.01	0.06
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
S. typhimurium/5 mmol l^{-1} 4-aminophenol	0	0.04	0.11	0.17	0.22	0.26	0.3	0.35	0.41	0.44	0.51
S. typhimurium/2.5 mmol l^{-1} 4-aminophenol	0	0.01	0.03	0.05	0.07	0.1	0.13	0.15	0.18	0.2	0.23
S. typhimurium/1.25 mmol l^{-1} 4-aminophenol	0	0	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.04	0.05
S. typhimurium/0.625 mmol l^{-1} 4-aminophenol	0	-0	0	0	0	0	0.01	0.01	0.01	0.01	0.02
S. typhimurium/0.313 mmol l^{-1} 4-aminophenol	0	-0	-0	-0	-0	-0	0	0	0	0.01	0.04
S. typhimurium/0.156 mmol l^{-1} 4-aminophenol	0	-0	-0	-0	-0	-0	-0	0	0	0.01	0.05
S. typhimurium/0.078 mmol l^{-1} 4-aminophenol	0	-0	-0	-0	-0	-0	-0	0	0	0.01	0.06
S. typhimurium/BHI control	0	-0	0	0	0	-0	-0	0	0	0.02	0.13
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
S.marcescens/5 mmol l^{-1} 4-aminophenol	0	0.04	0.11	0.17	0.22	0.26	0.3	0.35	0.41	0.45	0.5
S.marcescens/2.5 mmol l^{-1} 4-aminophenol	0	0.01	0.03	0.05	0.07	0.1	0.13	0.16	0.18	0.2	0.23
S.marcescens/1.25 mmol l^{-1} 4-aminophenol	0	0	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.04	0.05
S.marcescens/0.625 mmol l^{-1} 4-aminophenol	0	-0	0	0	0	0.01	0.01	0.01	0.01	0.01	0.02
S.marcescens/0.313 mmol l^{-1} 4-aminophenol	0	-0	-0	-0	-0	-0	0	0	0	0.01	0.03
S.marcescens/0.156 mmol l^{-1} 4-aminophenol	0	-0	-0	-0	-0	-0	-0	0	0	0.01	0.04
S.marcescens/0.078 mmol l^{-1} 4-aminophenol	0	-0	-0	-0	-0	-0	-0	-0	0	0.01	0.03
S.marcescens/BHI control	0	-0	-0	-0	-0	0	-0	0	0.01	0.01	0.1
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
E.faecalis/5 mmol l^{-1} 4-aminophenol	0	0.05	0.12	0.18	0.24	0.28	0.33	0.38	0.44	0.49	0.56
E.faecalis/2.5 mmol l^{-1} 4-aminophenol	0	0.01	0.03	0.05	0.07	0.1	0.12	0.15	0.17	0.19	0.23
E.faecalis/1.25 mmol l^{-1} 4-aminophenol	0	0	0.01	0.01	0.02	0.03	0.03	0.03	0.04	0.04	0.05
E.faecalis/0.625 mmol l^{-1} 4-aminophenol	0	0	0	0	0.01	0.01	0.01	0.01	0.01	0.02	0.03
E.faecalis/0.313 mmol l^{-1} 4-aminophenol	0	0	0	0	0	0	0	0.01	0.01	0.02	0.05
E.faecalis/0.156 mmol l^{-1} 4-aminophenol	0	-0	-0	-0	-0	0	0	0	0.01	0.03	0.09
E.faecalis/0.078 mmol l^{-1} 4-aminophenol	0	-0	-0	-0	-0	-0	0	0	0.01	0.01	0.07
E.faecalis/BHI control	0	-0	-0	-0	-0	-0	0	0	0.01	0.02	0.1
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
S.aureus/5 mmol l^{-1} 4-aminophenol	0	0.05	0.12	0.19	0.24	0.29	0.34	0.39	0.45	0.51	0.6
S.aureus/2.5 mmol l^{-1} 4-aminophenol	0	0.01	0.03	0.05	0.07	0.1	0.13	0.15	0.18	0.2	0.23
S.aureus/1.25 mmol l^{-1} 4-aminophenol	0	0	0.01	0.01	0.02	0.02	0.03	0.04	0.04	0.05	0.05
S.aureus/0.625 mmol l^{-1} 4-aminophenol	0	0	0	0	0.01	0.01	0.01	0.01	0.01	0.02	0.02
S.aureus/0.313 mmol l^{-1} 4-aminophenol	0	0	0	0	0	0	0	0	0	0	0.01
S.aureus/0.156 mmol l^{-1} 4-aminophenol	0	0	-0	-0	-0	-0	0.01	0.01	0.01	0.01	0
S.aureus/0.078 mmol l^{-1} 4-aminophenol	0	0	-0	-0	-0	-0	-0	-0	0	0.01	0
S.aureus/BHI control	0	-0	0	0	-0	0	-0	0	0.01	0.01	0.1

Appendix 2.1: Absorbance increases (405 nm) produced by various organisms in the presence of 4-aminophenols and *p*-phenylenediamine derivatives.

(continued)

	Time (mins)									
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
BHI broth/5 mmol l^{-1} 4-aminophenol	0	0.05	0.12	0.18	0.24	0.29	0.33	0.37	0.43	0.48	0.57
BHI broth/2.5 mmol l^{-1} 4-aminophenol	0	0.01	0.03	0.05	0.07	0.1	0.12	0.15	0.17	0.19	0.23
BHI broth/1.25 mmol l^{-1} 4-aminophenol	0	0	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.04	0.05
BHI broth/0.625 mmol l^{-1} 4-aminophenol	0	0	0	0	0.01	0.01	0.01	0.01	0.01	0.02	0.02
BHI broth/0.313 mmol l^{-1} 4-aminophenol	0	-0	-0	0	0	0	0	0	0	0	0
BHI broth/0.156 mmol l^{-1} 4-aminophenol	0	0	-0	-0	-0	-0	0	0	0	0	0
BHI broth/0.078 mmol l^{-1} 4-aminophenol	0	-0	-0	-0	-0	-0	-0	-0	-0	-0	0
BHI broth/BHI control	0	0	-0	-0	-0	-0	-0	-0	-0	-0	-0
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
E.coli/5 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0.01	0.03	0.05	0.06	0.08	0.1	0.12	0.13	0.15	0.17
E.coli/2.5 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0.01	0.02	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
E.coli/1.25 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.04	0.07
E.coli/0.625 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0	0.01	0.01	0.01	0.02	0.02	0.04	0.12
E.coli/0.313 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0	0.01	0.01	0.01	0.01	0.02	0.04	0.17
E.coli/0.156 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0	0	0.01	0.01	0.01	0.02	0.04	0.15
E.coli/0.078 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0	0	0	0.01	0.01	0.02	0.05	0.18
E.coli/BHI control	0	0	0	0	0	0	0	0.01	0.01	0.04	0.21
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
K. pneumoniae/5 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0.01	0.03	0.05	0.07	0.08	0.1	0.12	0.14	0.15	0.18
K. pneumoniae/2.5 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0.01	0.02	0.03	0.04	0.05	0.06	0.06	0.07	0.08	0.09
K. pneumoniae/1.25 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.04	0.05
K. pneumoniae/0.625 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0.01	0.01	0.01	0.01	0.02	0.02	0.03	0.08
K. pneumoniae/0.313 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0	0.01	0.01	0.01	0.01	0.02	0.03	0.12
K. pneumoniae/0.156 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0.01	0.01	0.01	0.01	0.01	0.01	0.03	0.13
K. pneumoniae/0.078 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0	0	0	0	0.01	0.01	0.02	0.11
K. pneumoniae/BHI control	0	-0	-0	-0	-0	-0	-0	0	0	0.03	0.12
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
E. cloacae/5 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0.01	0.03	0.05	0.06	0.08	0.1	0.12	0.14	0.15	0.18
E. cloacae/2.5 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0.01	0.02	0.03	0.03	0.04	0.05	0.06	0.07	0.08	0.1
E. cloacae/1.25 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.05	0.11
E. cloacae/0.625 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0.01	0.01	0.01	0.01	0.02	0.02	0.04	0.17
E. cloacae/0.313 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0	0.01	0.01	0.01	0.01	0.02	0.04	0.18
E. cloacae/0.156 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0.01	0.01	0.01	0.01	0.01	0.02	0.04	0.18
E. cloacae/0.078 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0	0	0	0.01	0.01	0.02	0.05	0.19
E. cloacae/BHI control	0	-0	-0	-0	-0	0	0	0.01	0.02	0.05	0.19
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
S. typhimurium/5 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0.01	0.03	0.05	0.06	0.08	0.1	0.12	0.14	0.15	0.18
S. typhimurium/2.5 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0.01	0.02	0.03	0.03	0.04	0.05	0.06	0.07	0.08	0.1
S. typhimurium/1.25 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.05	0.11
S. typhimurium/0.625 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0.01	0.01	0.01	0.02	0.02	0.03	0.05	0.17
S. typhimurium/0.313 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0	0.01	0.01	0.01	0.01	0.03	0.06	0.2
S. typhimurium/0.156 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0	0	0.01	0.01	0.01	0.03	0.06	0.21
S. typhimurium/0.078 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0	0	0	0.01	0.01	0.03	0.07	0.21
S. typhimurium/BHI control	0	-0	-0	-0	-0	-0	-0	0	0.02	0.05	0.2
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
S.marcescens/5 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0.02	0.03	0.05	0.07	0.09	0.1	0.12	0.14	0.16	0.18
S.marcescens/2.5 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0.01	0.02	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
S.marcescens/1.25 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0.01	0.01	0.02	0.02	0.03	0.03	0.03	0.04	0.06
S.marcescens/0.625 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0.01	0.01	0.01	0.02	0.02	0.02	0.03	0.07
S.marcescens/0.313 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0	0	0.01	0.01	0.01	0.02	0.03	0.09
S.marcescens/0.156 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0	0	0	0	0.01	0.01	0.02	0.1
S.marcescens/0.078 mmol l^{-1} 4-amino-2,6-dibromophenol	0	-0	-0	0	0	0	0	0	0.01	0.03	0.11
S.marcescens/BHI control	0	-0	-0	-0	-0	0	0	0	0.01	0.03	0.13
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
E.faecalis/5 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0.01	0.03	0.05	0.07	0.09	0.1	0.12	0.14	0.16	0.18
E.faecalis/2.5 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0.01	0.02	0.03	0.04	0.04	0.05	0.06	0.07	0.08	0.1
E.faecalis/1.25 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0.01	0.01	0.02	0.02	0.03	0.03	0.05	0.06	0.14
E.faecalis/0.625 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0.01	0.01	0.01	0.02	0.02	0.03	0.05	0.08	0.25
E.faecalis/0.313 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0.01	0.01	0.01	0.01	0.02	0.05	0.1	0.31
E.faecalis/0.156 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0	0.01	0.01	0.01	0.02	0.05	0.11	0.33
E.faecalis/0.078 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0	0	0.01	0.01	0.02	0.05	0.11	0.33
E.faecalis/BHI control	0	0	0	0	0	0	0	0.01	0.03	0.05	0.31

Appendix 2.1: Absorbance increases (405 nm) produced by various organisms in the presence of 4-aminophenols and *p*-phenylenediamine derivatives. (continued)	Time (mins)										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
S.aureus/5 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0.01	0.03	0.05	0.07	0.09	0.1	0.12	0.14	0.16	0.18
S.aureus/2.5 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0.01	0.02	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
S.aureus/1.25 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0.01	0.01	0.02	0.02	0.03	0.03	0.03	0.04	0.04
S.aureus/0.625 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02
S.aureus/0.313 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
S.aureus/0.156 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0.01	0.06	0	0.01	0.01	0.01	0.01	0.01	0.01
S.aureus/0.078 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0	0	0	0	0	0	0	0
S.aureus/BHI control	0	-0	0	0	0	0	0	0	0.04	0.08	0.1
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
BHI broth/5 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0.01	0.03	0.05	0.07	0.09	0.11	0.12	0.14	0.16	0.18
BHI broth/2.5 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0.01	0.02	0.03	0.03	0.04	0.05	0.06	0.07	0.08	0.09
BHI broth/1.25 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0.01	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.04	0.04
BHI broth/0.625 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02
BHI broth/0.313 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0	0.01	0.01	0.01	0.01	0.01	0.01	0.01
BHI broth/0.156 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
BHI broth/0.078 mmol l^{-1} 4-amino-2,6-dibromophenol	0	0	0	0	0	0	0	0	0	0.01	0.01
BHI broth/BHI control	0	-0	-0	-0	-0	-0	-0	-0	-0	-0	-0
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
E.coli/5 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.02	0.06	0.11	0.17	0.24	0.31	0.38	0.47	0.54	0.66
E.coli/2.5 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.01	0.03	0.05	0.07	0.1	0.12	0.15	0.18	0.2	0.25
E.coli/1.25 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.01	0.02	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.1
E.coli/0.625 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0.01	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.04
E.coli/0.313 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02
E.coli/0.156 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.03
E.coli/0.078 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0	0	0	0.01	0.01	0.01	0.01	0.01	0.04
E.coli/BHI control	0	0	0	-0	0	0	0	0	0.01	0.01	0.12
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
K. pneumoniae/5 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.02	0.06	0.11	0.17	0.24	0.31	0.39	0.47	0.55	0.68
K. pneumoniae/2.5 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.01	0.03	0.05	0.07	0.1	0.12	0.15	0.18	0.21	0.25
K. pneumoniae/1.25 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.01	0.02	0.02	0.03	0.04	0.05	0.07	0.08	0.09	0.11
K. pneumoniae/0.625 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0.01	0.01	0.02	0.02	0.03	0.03	0.03	0.04	0.05
K. pneumoniae/0.313 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.03
K. pneumoniae/0.156 mmol l-1 4-amino-2,6-dichlorophenol	0	0	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.03
K. pneumoniae/0.078 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0	0	0	0.01	0.01	0.01	0.01	0.01	0.04
K. pneumoniae/BHI control	0	-0	-0	-0	-0	-0	-0	-0	0	0.02	0.11
	0	***30***	***60***	***90***	***120***	***150***	***180***	***210***	***240***	***270***	***300***
E. cloacae/5 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.02	0.06	0.11	0.17	0.24	0.31	0.38	0.47	0.55	0.67
E. cloacae/2.5 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.01	0.03	0.05	0.07	0.1	0.12	0.15	0.18	0.21	0.26
E. cloacae/1.25 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.01	0.02	0.02	0.03	0.05	0.06	0.07	0.08	0.09	0.11
E. cloacae/0.625 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.04	0.05
E. cloacae/0.313 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.03	0.05
E. cloacae/0.156 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.08
E. cloacae/0.078 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0	0	0	0.01	0.01	0.01	0.01	0.02	0.08
E. cloacae/BHI control	0	0	0	0	0	0	0	0	0.01	0.01	0.11
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
S. typhimurium/5 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.02	0.06	0.11	0.17	0.24	0.31	0.38	0.47	0.55	0.68
S. typhimurium/2.5 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.01	0.03	0.05	0.07	0.1	0.13	0.15	0.18	0.21	0.26
S. typhimurium/1.25 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.01	0.02	0.02	0.03	0.05	0.06	0.07	0.08	0.09	0.11
S. typhimurium/0.625 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.04	0.05
S. typhimurium/0.313 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.03	0.05
S. typhimurium/0.156 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0	0.01	0.01	0.01	0.01	0.01	0.02	0.03	0.07
S. typhimurium/0.078 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0	0	0.01	0.01	0.01	0.01	0.01	0.03	0.1
S. typhimurium/BHI control	0	0	0	0	0	0	0	0	0.01	0.01	0.14
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
S.marcescens/5 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.02	0.06	0.11	0.17	0.23	0.3	0.37	0.46	0.54	0.66
S.marcescens/2.5 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.01	0.03	0.05	0.07	0.1	0.12	0.15	0.18	0.21	0.26
S.marcescens/1.25 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.01	0.02	0.02	0.03	0.04	0.06	0.07	0.08	0.09	0.11
S.marcescens/0.625 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0.01	0.01	0.02	0.02	0.03	0.04	0.04	0.05	0.06
S.marcescens/0.313 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.03	0.05
S.marcescens/0.156 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.06
S.marcescens/0.078 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0	0	0	0.01	0.01	0.01	0.01	0.02	0.06
S.marcescens/BHI control	*0*	*0*	*0*	*-0*	*-0*	*0*	*0*	*0*	*0.01*	*0.02*	*0.14*

Appendix 2.1: Absorbance increases (405 nm) produced by various organisms in the presence of 4-aminophenols and *p*-phenylenediamine derivatives.

(continued)

	Time (mins)										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
E.faecalis/5 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.02	0.06	0.11	0.17	0.24	0.31	0.38	0.47	0.55	0.67
E.faecalis/2.5 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.02	0.03	0.05	0.07	0.1	0.12	0.15	0.17	0.2	0.25
E.faecalis/1.25 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.01	0.02	0.02	0.03	0.04	0.05	0.07	0.08	0.09	0.1
E.faecalis/0.625 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.04	0.06
E.faecalis/0.313 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0.01	0.01	0.01	0.01	0.02	0.02	0.03	0.05	0.12
E.faecalis/0.156 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.03	0.1
E.faecalis/0.078 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0	0	0.01	0.01	0.01	0.01	0.02	0.03	0.09
E.faecalis/BHI control	0	0	0	0	0	0	0	0.01	0.01	0.02	0.12
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
S.aureus/5 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.03	0.07	0.11	0.17	0.24	0.31	0.38	0.46	0.54	0.66
S.aureus/2.5 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.01	0.03	0.05	0.07	0.09	0.12	0.14	0.17	0.19	0.24
S.aureus/1.25 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.01	0.02	0.02	0.03	0.04	0.05	0.06	0.08	0.09	0.1
S.aureus/0.625 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.04	0.05	0.05
S.aureus/0.313 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
S.aureus/0.156 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.01	0.01	0.06	0	0.01	0.01	0.01	0.01	0.01	0.01
S.aureus/0.078 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0	0	0	0	0	0	0	0	0
S.aureus/BHI control	0	0	0	0	0	0	0	0	0.04	0.08	0.12
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
BHI broth/5 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.03	0.07	0.12	0.18	0.25	0.32	0.4	0.48	0.56	0.68
BHI broth/2.5 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.01	0.03	0.05	0.07	0.1	0.12	0.14	0.17	0.2	0.24
BHI broth/1.25 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.11
BHI broth/0.625 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.01	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.04	0.05
BHI broth/0.313 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.03
BHI broth/0.156 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02
BHI broth/0.078 mmol l^{-1} 4-amino-2,6-dichlorophenol	0	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
BHI broth/BHI control	0	0	0	0	0	0	0	0	-0	0	0
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
E.coli/5 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0.01	0.02	0.03	0.05	0.06	0.08	0.09	0.11	0.12	0.14
E.coli/2.5 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0.01	0.02	0.02	0.03	0.03	0.04	0.05	0.05	0.06
E.coli/1.25 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.03
E.coli/0.625 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0	0	0	0.01	0.01	0.01	0.01	0.03
E.coli/0.313 mmol l^{-1} diethyl-*p*-phenylenediamine	0	-0	0	0	0	0	0	0	0.01	0.01	0.03
E.coli/0.156 mmol l^{-1} diethyl-*p*-phenylenediamine	0	-0	0	0	0	0	0	0	0	0.01	0.04
E.coli/0.078 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0	0	0	0	0	0.01	0.01	0.05
E.coli/BHI control	0	-0	-0	-0	-0	-0	0	0	0	0.01	0.14
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
K. pneumoniae/5 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0.01	0.02	0.03	0.05	0.06	0.07	0.09	0.1	0.12	0.14
K. pneumoniae/2.5 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0.01	0.02	0.02	0.03	0.04	0.04	0.05	0.06	0.07
K. pneumoniae/1.25 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.03	0.04
K. pneumoniae/0.625 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0	0	0.01	0.01	0.01	0.01	0.01	0.04
K. pneumoniae/0.313 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0	0	0	0	0	0.01	0.01	0.03
K. pneumoniae/0.156 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0	0	0	0	0	0.01	0.01	0.03
K. pneumoniae/0.078 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0	0	0	0	0	0.01	0.01	0.06
K. pneumoniae/BHI control	0	-0	-0	-0	-0	-0	-0	-0	0	0	0.14
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
E. cloacae/5 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0.01	0.02	0.04	0.05	0.06	0.08	0.09	0.11	0.12	0.14
E. cloacae/2.5 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0.01	0.02	0.02	0.03	0.04	0.04	0.05	0.06	0.09
E. cloacae/1.25 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.04	0.1
E. cloacae/0.625 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0	0	0.01	0.01	0.01	0.01	0.03	0.11
E. cloacae/0.313 mmol l^{-1} diethyl-*p*-phenylenediamine	0	-0	0	0	0	0	0	0	0.01	0.02	0.11
E. cloacae/0.156 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0	0	0	0	0	0.01	0.02	0.11
E. cloacae/0.078 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0	0	0	0	0	0.01	0.02	0.1
E. cloacae/BHI control	0	-0	-0	-0	-0	0	0	0	0	0.01	0.12
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
S. typhimurium/5 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0.01	0.02	0.04	0.05	0.06	0.08	0.09	0.11	0.12	0.15
S. typhimurium/2.5 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0.01	0.02	0.02	0.03	0.03	0.04	0.05	0.06	0.07
S. typhimurium/1.25 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0.01	0.01	0.01	0.02	0.02	0.02	0.03	0.06
S. typhimurium0.625 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0	0.01	0.01	0.01	0.01	0.02	0.02	0.08
S. typhimurium/0.313 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0.01	0.01	0.01	0.01	0.01	0.02	0.03	0.1
S. typhimurium/0.156 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0	0	0	0	0.01	0.01	0.03	0.09
S. typhimurium/0.078 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0	0	0	0	0.01	0.01	0.02	0.09
S. typhimurium/BHI control	0	0	0	0	0	0	0	0	0.01	0.02	0.16

Appendix 2.1: Absorbance increases (405 nm) produced by various organisms in the presence of 4-aminophenols and *p*-phenylenediamine derivatives.

(continued)

	Time (mins)										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
S.marcescens/5 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0.01	0.02	0.04	0.05	0.07	0.08	0.1	0.11	0.13	0.15
S.marcescens/2.5 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0.01	0.02	0.02	0.03	0.04	0.04	0.05	0.06	0.08
S.marcescens/1.25 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.03	0.06
S.marcescens/0.625 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0	0.01	0.01	0.01	0.01	0.02	0.03	0.08
S.marcescens/0.313 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0	0	0	0	0.01	0.01	0.02	0.08
S.marcescens/0.156 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0	0	0	0	0.01	0.01	0.03	0.1
S.marcescens/0.078 mmol l^{-1} diethyl-*p*-phenylenediamine	0	-0	-0	0	0	0	0	0	0.01	0.02	0.08
S.marcescens/BHI control	0	0	0	0	0	0	0	0	0.01	0.02	0.11
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
E.faecalis/5 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0.01	0.03	0.04	0.05	0.07	0.08	0.1	0.11	0.13	0.15
E.faecalis/2.5 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0.01	0.01	0.02	0.03	0.03	0.04	0.04	0.05	0.06	0.07
E.faecalis/1.25 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0.01	0.01	0.01	0.01	0.02	0.02	0.03	0.05	0.12
E.faecalis/0.625 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0.01	0.01	0.01	0.01	0.02	0.03	0.05	0.14
E.faecalis/0.313 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0	0	0.01	0.01	0.01	0.02	0.04	0.14
E.faecalis/0.156 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0	0	0	0.01	0.01	0.02	0.03	0.1
E.faecalis/0.078 mmol l^{-1} diethyl-p-phenylenediamine	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0.01*	*0.02*	*0.03*	*0.08*
E.faecalis/BHI control	0	0	0	0	0	0	0	0.01	0.01	0.02	0.1
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
S.aureus/5 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0.01	0.02	0.04	0.05	0.07	0.08	0.09	0.11	0.12	0.15
S.aureus/2.5 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0.01	0.01	0.02	0.02	0.03	0.04	0.04	0.05	0.06	0.07
S.aureus/1.25 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.03	0.03
S.aureus.0.625 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02
S.aureus/0.313 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0	0.01	0.01	0.01	0.01	0.01	0.01	0.01
S.aureus/0.156 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0	0	0.01	0.01	0.01	0.01	0.01	0.01
S.aureus/0.078 mmol l^{-1} diethyl-*p*-phenylenediamine	0	-0	0	0	0	0	0	0	0	0	0
S.aureus/BHI control	0	0	0	0	0	0	0	0	0.04	0.08	0.1
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
BHI broth/5 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0.01	0.03	0.05	0.08	0.07	0.09	0.1	0.12	0.13	0.16
BHI broth/2.5 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0.01	0.01	0.02	0.02	0.03	0.04	0.04	0.05	0.06	0.07
BHI broth/1.25 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.03
BHI broth/0.625 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02
BHI broth/0.313 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0	0	0.01	0.01	0.01	0.01	0.01	0.01
BHI broth/0.156 mmol l^{-1} diethyl-*p*-phenylenediamine	0	0	0	0	0	0	0	0	0.01	0.01	0.01
BHI broth/0.078 mmol l^{-1} diethyl-*p*-phenylenediamine	0	-0	0	0	0	0	0	0	0	0	0
BHI broth/BHI control	0	-0	-0	-0	-0	-0	-0	-0	-0	-0	-0
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
E.coli/5 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0.03	0.07	0.12	0.16	0.21	0.26	0.29	0.24	0.23	0.49
E.coli/2.5 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0.01	0.03	0.05	0.07	0.09	0.11	0.13	0.14	0.16	0.19
E.coli/1.25 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0.01	0.01	0.02	0.03	0.04	0.04	0.05	0.05	0.06	0.07
E.coli/0.625 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	-0	0	0	0.01	0.01	0.01	0.02	0.02	0.02	0.04
E.coli/0.313 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0	0	0	0.01	0.01	0.01	0.01	0.02	0.09
E.coli/0.156 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0	0	0	0	0.01	0.01	0.01	0.03	0.12
E.coli/0.078 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0	0	0	0	0	0.01	0.01	0.03	0.14
E.coli/BHI control	0	-0	-0	-0	0	0	0	0	0.01	0.04	0.15
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
K. pneumoniae/5 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0.03	0.07	0.12	0.16	0.21	0.26	0.3	0.26	0.25	0.5
K. pneumoniae/2.5 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0.01	0.03	0.05	0.07	0.09	0.11	0.13	0.14	0.16	0.19
K. pneumoniae/1.255 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0.01	0.02	0.03	0.04	0.04	0.05	0.05	0.06	0.07
K. pneumoniae/0.625 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0	0.01	0.01	0.01	0.02	0.02	0.02	0.03	0.04
K. pneumoniae/0.313 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0	0	0	0.01	0.01	0.01	0.01	0.02	0.06
K. pneumoniae/0.156 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0	0	0	0.01	0.01	0.01	0.01	0.02	0.07
K. pneumoniae/0.078 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0	0	0	0	0	0.01	0.01	0.02	0.1
K. pneumoniae/BHI control	0	-0	-0	-0	-0	-0	0	0	0.01	0.01	0.14
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
E. cloacae/5 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0.03	0.07	0.11	0.16	0.21	0.25	0.29	0.23	0.22	0.42
E. cloacae/2.5 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0.01	0.03	0.05	0.07	0.09	0.11	0.13	0.14	0.16	0.19
E. cloacae/1.25 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0.01	0.02	0.03	0.03	0.04	0.05	0.06	0.06	0.1
E. cloacae/0.625 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0	0.01	0.01	0.01	0.02	0.02	0.03	0.04	0.13
E. cloacae/0.313 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	-0	0	0	0	0.01	0.01	0.01	0.02	0.04	0.16
E. cloacae/0.156 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0	0	0	0	0.01	0.01	0.02	0.03	0.14
E. cloacae/0.078 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0	0	0	0	0	0.01	0.01	0.04	0.15
E. cloacae/BHI control	0	-0	-0	-0	-0	-0	0	0	0.01	0.04	0.17

Appendix 2.1: Absorbance increases (405 nm) produced by various organisms in the presence of 4-aminophenols and *p*-phenylenediamine derivatives. (continued)

	Time (mins)										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
S. typhimurium/5 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0.03	0.07	0.11	0.16	0.21	0.26	0.3	0.25	0.23	0.64
S. typhimurium/2.5 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0.01	0.03	0.05	0.07	0.09	0.11	0.13	0.14	0.16	0.19
S. typhimurium/1.25 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0.01	0.02	0.03	0.03	0.04	0.05	0.05	0.06	0.09
S. typhimurium/0.625 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0	0.01	0.01	0.01	0.02	0.02	0.03	0.03	0.1
S. typhimurium/0.313 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	-0	0	0	0	0.01	0.01	0.01	0.02	0.04	0.16
S. typhimurium/0.156 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0	0	0	0	0.01	0.01	0.02	0.04	0.14
S. typhimurium/0.078 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0	0	0	0	0.01	0.01	0.02	0.04	0.15
S. typhimurium/BHI control	0	-0	-0	-0	-0	0	0	0.01	0.02	0.04	0.15
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
S.marcescens/5 mmol l^{-1} dimethyl-p-phenylenediamine	0	0.03	0.07	0.11	0.16	0.21	0.25	0.29	0.25	0.27	0.75
S.marcescens/2.5 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0.01	0.03	0.05	0.07	0.09	0.11	0.12	0.14	0.16	0.19
S.marcescens/1.25 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0.01	0.02	0.02	0.03	0.04	0.04	0.05	0.06	0.07
S.marcescens/0.625 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0	0.01	0.01	0.01	0.02	0.02	0.02	0.03	0.07
S.marcescens/0.313 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0	0	0	0.01	0.01	0.01	0.01	0.02	0.07
S.marcescens/0.156 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	-0	0	0	0	0	0	0	0.01	0.02	0.08
S.marcescens/0.078 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0	0	0	0	0	0	0.01	0.02	0.09
S.marcescens/BHI control	0	-0	-0	-0	-0	-0	0	0	0.01	0.02	0.1
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
E.faecalis/5 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0.03	0.08	0.12	0.17	0.21	0.26	0.3	0.26	0.25	0.61
E.faecalis/2.5 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0.01	0.03	0.05	0.07	0.09	0.1	0.12	0.14	0.16	0.19
E.faecalis1.25 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0.01	0.02	0.03	0.03	0.04	0.05	0.06	0.07	0.12
E.faecalis/0.625 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0.01	0.01	0.01	0.01	0.02	0.02	0.04	0.06	0.16
E.faecalis/0.313 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0	0	0.01	0.01	0.01	0.02	0.03	0.05	0.15
E.faecalis/0.156 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0	0	0	0	0.01	0.01	0.02	0.05	0.15
E.faecalis/0.078 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0	0	0	0	0.01	0.01	0.02	0.04	0.14
E.faecalis/BHI control	0	-0	0	-0	0	0	0	0.01	0.02	0.04	0.15
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
S.aureus/5 mmol l^{-1} dimethyl-p-phenylenediamine	0	0.03	0.07	0.12	0.17	0.21	0.26	0.31	0.28	0.24	0.34
S.aureus/2.5 mmol l^{-1} dimethyl-p-phenylenediamine	0	0.01	0.03	0.05	0.07	0.09	0.1	0.12	0.14	0.15	0.18
S.aureus/1.25 mmol l^{-1} dimethyl-p-phenylenediamine	0	0.01	0.01	0.02	0.03	0.03	0.04	0.05	0.05	0.06	0.07
S.aureus/0.625 mmol l^{-1} dimethyl-p-phenylenediamine	0	0	0	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.03
S.aureus/0.313 mmol l^{-1} dimethyl-p-phenylenediamine	0	0	0	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
S.aureus/0.156 mmol l^{-1} dimethyl-p-phenylenediamine	0	0	0	0	0	0.01	0.01	0.01	0.01	0.01	0.01
S.aureus/0.078 mmol l^{-1} dimethyl-p-phenylenediamine	0	0	0	0	0	0	0	0	0	0	0
S.aureus/BHI control	0	0	0	0	0	0	0	0	0.04	0.08	0.1
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
BHI broth/5 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0.03	0.08	0.12	0.17	0.22	0.27	0.31	0.28	0.28	0.46
BHI broth/2.5 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0.01	0.03	0.05	0.07	0.09	0.11	0.12	0.14	0.16	0.18
BHI broth/1.25 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0.01	0.02	0.03	0.03	0.04	0.04	0.05	0.05	0.06
BHI broth/0.625 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02
BHI broth/0.313 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0	0	0.01	0.01	0.01	0.01	0.01	0.01	0.01
BHI broth/0.156 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
BHI broth/0.078 mmol l^{-1} dimethyl-*p*-phenylenediamine	0	0	0	0	0	0	0	0	0	0	0
BHI broth/BHI control	0	0	0	0	0	-0	-0	-0	-0	0	-0

Appendix 2.2: Absorbance increases (405 nm) produced by various organisms in the presence of various naphthols.

	Time (mins)									
BHI 3,5-dihydroxy-2-naphthoic acid										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	-0.002	-0.003	-0.001	-0.002	-0.001	-0.001	-0.001	0.000	0.001	0
0.5 mmol l^{-1}	0	0	0	0	0	0.001	0.001	0.001	0.001	0.001	0.002
0.25 mmol l^{-1}	0	0	-0.001	-0.001	-0.001	0	0	0	0.000	0.001	0.002
0.125 mmol l^{-1}	0	0	-0.001	-0.001	-0.001	0	0	0	0.000	0	0
0.0625 mmol l^{-1}	0	0	0	0	0	0	0.0015	0.002	0.001	0.001	0.002
BHI control	0	0	-0.001	-0.001	-0.001	-0.001	-0.002	-0.002	-0.002	-0.002	-0.001
S.aureus 3,5-dihydroxy-2-naphthoic acid										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	-0.002	-0.002	-1E-03	0.002	0.005	0.0165	0.028	0.061	0.117	0.209
0.5 mmol l^{-1}	0	-0.002	-0.001	0	0.001	0.004	0.028	0.052	0.102	0.176	0.289
0.25 mmol l^{-1}	0	-0.001	-0.001	0	0.001	0.005	0.0265	0.048	0.102	0.185	0.305
0.125 mmol l^{-1}	0	-0.001	0.003	0.003	0.004	0.006	0.029	0.052	0.111	0.195	0.314
0.0625 mmol l^{-1}	0	-0.002	-0.002	-0.001	0	0.004	0.0275	0.051	0.108	0.197	0.323
BHI control	0	-0.002	-0.002	-1E-03	0	0.004	0.028	0.052	0.110	0.198	0.321
E.faecalis 3,5-dihydroxy-2-naphthoic acid										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0	0.001	0.002	0.005	0.012	0.0345	0.057	0.119	0.214	0.378
0.5 mmol l^{-1}	0	-0.001	0.001	0.002	0.005	0.012	0.0425	0.073	0.162	0.294	0.476
0.25 mmol l^{-1}	0	-0.001	0	0.001	0.004	0.011	0.0495	0.088	0.205	0.407	0.624
0.125 mmol l^{-1}	0	-0.001	0	0.001	0.004	0.012	0.0575	0.103	0.228	0.409	0.603
0.0625 mmol l^{-1}	0	0	0	0.002	0.006	0.015	0.0665	0.118	0.252	0.454	0.662
BHI control	0	-0.001	-0.001	0	0.004	0.012	0.063	0.114	0.252	0.46	0.654
S .marcescens 3,5-dihydroxy-2-naphthoic acid										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	-0.002	0	1E-03	0.005	0.013	0.1265	0.24	0.339	0.536	0.656
0.5 mmol l^{-1}	0	0	1E-03	0.002	0.005	0.01	0.073	0.136	0.222	0.34	0.459
0.25 mmol l^{-1}	0	-1E-03	0	0	0.002	0.007	0.0415	0.076	0.162	0.263	0.364
0.125 mmol l^{-1}	0	0.001	0.001	0.002	0.004	0.01	0.0375	0.065	0.145	0.247	0.34
0.0625 mmol l^{-1}	0	-0.001	0	1E-03	0.004	0.011	0.0425	0.074	0.154	0.256	0.346
BHI control	0	0	0.001	0.002	0.004	0.01	0.037	0.064	0.134	0.227	0.322
S .typhimurium 3,5-dihydroxy-2-naphthoic acid										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	-0.002	0	0.002	0.008	0.024	0.091	0.158	0.249	0.361	0.482
0.5 mmol l^{-1}	0	-0.001	0.001	0.003	0.008	0.023	0.0895	0.156	0.257	0.34	0.428
0.25 mmol l^{-1}	0	-0.002	-0.001	0.001	0.006	0.02	0.082	0.144	0.243	0.324	0.413
0.125 mmol l^{-1}	0	-0.001	0	0.002	0.008	0.022	0.0835	0.145	0.241	0.316	0.394
0.0625 mmol l^{-1}	0	-0.001	-0.001	0.002	0.007	0.022	0.087	0.152	0.253	0.316	0.391
BHI control	0	-0.001	0	0.002	0.008	0.023	0.088	0.153	0.255	0.31	0.384
E. cloacae 3,5-dihydroxy-2-naphthoic acid										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	-0.001	0.001	0.002	0.006	0.014	0.0705	0.127	0.251	0.349	0.456
0.5 mmol l^{-1}	0	0	0.001	0.003	0.007	0.016	0.0745	0.133	0.249	0.37	0.549
0.25 mmol l^{-1}	0	-0.002	-0.001	1E-03	0.004	0.016	0.077	0.138	0.268	0.401	0.578
0.125 mmol l^{-1}	0	-0.002	-0.001	0.001	0.005	0.018	0.0945	0.171	0.287	0.457	0.586
0.0625 mmol l^{-1}	0	-0.003	-0.001	0.001	0.006	0.02	0.1065	0.193	0.304	0.503	0.585
BHI control	0	-0.002	-0.001	0.001	0.006	0.02	0.1035	0.187	0.300	0.497	0.585

Appendix 2.2: Absorbance increases (405 nm) produced by various organisms in the presence of various naphthols.

	Time (mins)									
K.pneumoniae 3,5-dihydroxy-2-naphthoic acid										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.003	0.004	0.006	0.011	0.018	0.058	0.098	0.194	0.315	0.494
0.5 mmol l^{-1}	0	0	1E-03	0.003	0.006	0.013	0.084	0.155	0.290	0.452	0.593
0.25 mmol l^{-1}	0	-0.001	0	0.002	0.006	0.015	0.092	0.169	0.305	0.489	0.688
0.125 mmol l^{-1}	0	0	0.001	0.003	0.007	0.016	0.091	0.166	0.299	0.488	0.678
0.0625 mmol l^{-1}	0	-0.003	-0.001	0.001	0.005	0.016	0.0935	0.171	0.329	0.506	0.708
BHI control	0	-0.003	-0.001	0	0.006	0.017	0.101	0.185	0.335	0.488	0.587
E. coli 3,5-dihydroxy-2-naphthoic acid										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	-0.001	0.003	1E-03	0.004	0.01	0.0485	0.087	0.176	0.273	0.397
0.5 mmol l^{-1}	0	0	0	0.001	0.004	0.01	0.045	0.08	0.158	0.247	0.347
0.25 mmol l^{-1}	0	0	0	1E-03	0.003	0.01	0.0465	0.083	0.163	0.258	0.367
0.125 mmol l^{-1}	0	-0.005	-0.004	-0.004	0	0.008	0.053	0.098	0.184	0.289	0.423
0.0625 mmol l^{-1}	0	0	0	1E-03	0.005	0.014	0.0665	0.119	0.209	0.322	0.458
BHI control	0	-0.001	-0.001	1E-03	0.004	0.015	0.073	0.131	0.223	0.346	0.46
BHI 5[[(1-hydroxy-2-naphthyl)-carbonyl]octadecylamino]-isophthalic acid										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0	0	0	0	0	0	0	0.001	0	0.002
0.5 mmol l^{-1}	0	0	-0.001	-0.001	-0.001	-0.001	-0.001	-0.001	-0.001	-0.001	-0.001
0.25 mmol l^{-1}	0	0.001	0	0	0	0	0	0	0.000	0.001	0.001
0.125 mmol l^{-1}	0	0.054	0.038	0.042	0.037	0.043	0.0435	0.044	0.043	0.042	0.045
0.0625 mmol l^{-1}	0	-0.001	-0.001	-0.001	-0.002	-0.001	-0.001	-0.001	-0.001	-0.001	-0.001
BHI control	0	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.002
S.aureus 5[[(1-hydroxy-2-naphthyl)-carbonyl]octadecylamino]-isophthalic acid										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.001	0.002	0.002	0.002	0.002	0.004	0.006	0.012	0.025	0.046
0.5 mmol l^{-1}	0	0	0	0.001	0.001	0.002	0.0055	0.009	0.019	0.037	0.057
0.25 mmol l^{-1}	0	0.003	0.004	0.004	0.004	0.006	0.0115	0.017	0.035	0.06	0.082
0.125 mmol l^{-1}	0	-0.002	-0.002	-0.001	-0.001	0.001	0.01	0.019	0.045	0.078	0.109
0.0625 mmol l^{-1}	0	0.002	0.003	0.003	0.004	0.006	0.02	0.034	0.074	0.124	0.175
BHI control	0	-0.003	-0.002	0.001	0.001	0.005	0.032	0.059	0.124	0.219	0.367
E.faecalis 5[[(1-hydroxy-2-naphthyl)-carbonyl]octadecylamino]-isophthalic acid										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.001	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003
0.5 mmol l^{-1}	0	0.001	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.003	0.003
0.25 mmol l^{-1}	0	0.004	0.005	0.005	0.006	0.006	0.008	0.01	0.013	0.015	0.02
0.125 mmol l^{-1}	0	-0.001	-0.001	-0.001	0	0.002	0.0085	0.015	0.025	0.039	0.049
0.0625 mmol l^{-1}	0	0.005	0.005	0.006	0.007	0.01	0.0255	0.041	0.066	0.102	0.166
BHI control	0	-0.001	-0.001	0.002	0.008	0.02	0.085	0.15	0.283	0.486	0.726
S.marcescens 5[[(1-hydroxy-2-naphthyl)-carbonyl]octadecylamino]-isophthalic acid										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.001	0.002	0.002	0.004	0.008	0.0235	0.039	0.088	0.174	0.274
0.5 mmol l^{-1}	0	0	1E-03	0.002	0.004	0.008	0.0265	0.045	0.097	0.184	0.271
0.25 mmol l^{-1}	0	0.004	0.004	0.005	0.007	0.013	0.0365	0.06	0.122	0.211	0.299
0.125 mmol l^{-1}	0	-0.002	-0.002	-0.001	0.001	0.006	0.026	0.046	0.101	0.177	0.261
0.0625 mmol l^{-1}	0	0	0.001	0.002	0.003	0.008	0.027	0.046	0.105	0.185	0.267
BHI control	0	-0.004	-0.003	-0.002	0.001	0.008	0.043	0.078	0.173	0.266	0.348

Appendix 2.2: Absorbance increases (405 nm) produced by various organisms in the presence of various naphthols.											
		Time	(mins)								
S.typhimurium 5[[(1-hydroxy-2-naphthyl)-carbonyl]octadecylamino]-isophthalic acid											
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.001	0.002	0.005	0.012	0.031	0.1115	0.192	0.306	0.369	0.428
0.5 mmol l^{-1}	0	0.001	0.002	0.004	0.011	0.028	0.097	0.166	0.265	0.328	0.391
0.25 mmol l^{-1}	0	0.001	0.003	0.005	0.012	0.029	0.096	0.163	0.262	0.33	0.396
0.125 mmol l^{-1}	0	-0.005	-0.004	-0.002	0.004	0.021	0.084	0.147	0.240	0.312	0.377
0.0625 mmol l^{-1}	0	0.002	0.003	0.006	0.012	0.029	0.0935	0.158	0.254	0.313	0.375
BHI control	0	-0.002	-0.002	0.001	0.007	0.024	0.0915	0.159	0.263	0.322	0.396
E.cloacae 5[[(1-hydroxy-2-naphthyl)-carbonyl]octadecylamino]-isophthalic acid											
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.001	0.003	0.005	0.009	0.021	0.0855	0.15	0.279	0.472	0.574
0.5 mmol l^{-1}	0	-0.001	0.006	0.003	0.015	0.022	0.0995	0.177	0.311	0.51	0.595
0.25 mmol l^{-1}	0	0.003	0.005	0.007	0.012	0.027	0.1	0.173	0.296	0.489	0.567
0.125 mmol l^{-1}	0	-0.001	0	0.002	0.007	0.022	0.093	0.164	0.303	0.462	0.575
0.0625 mmol l^{-1}	0	0.002	0.004	0.006	0.011	0.026	0.098	0.17	0.299	0.497	0.578
BHI control	0	0	0.001	0.005	0.01	0.026	0.112	0.198	0.327	0.519	0.594
K.pneumoniae 5[[(1-hydroxy-2-naphthyl)-carbonyl]octadecylamino]-isophthalic acid											
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.002	0.004	0.005	0.01	0.02	0.0705	0.121	0.249	0.397	0.551
0.5 mmol l^{-1}	0	0.002	0.003	0.006	0.012	0.023	0.0725	0.122	0.222	0.348	0.51
0.25 mmol l^{-1}	0	0.002	0.004	0.006	0.01	0.021	0.0665	0.112	0.204	0.338	0.535
0.125 mmol l^{-1}	0	-0.004	-0.002	-0.001	0.003	0.013	0.0565	0.1	0.190	0.322	0.51
0.0625 mmol l^{-1}	0	0.001	0.003	0.005	0.009	0.019	0.062	0.105	0.210	0.344	0.546
BHI control	0	-0.003	-0.003	-0.001	0.005	0.02	0.104	0.188	0.346	0.533	0.761
E.coli 5[[(1-hydroxy-2-naphthyl)-carbonyl]octadecylamino]-isophthalic acid											
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.003	0.003	0.002	0.005	0.015	0.073	0.131	0.244	0.375	0.488
0.5 mmol l^{-1}	0	-0.005	-0.005	-0.005	-0.001	0.009	0.0635	0.118	0.220	0.346	0.477
0.25 mmol l^{-1}	0	-0.002	-0.001	0	0.004	0.013	0.0645	0.116	0.219	0.353	0.456
0.125 mmol l^{-1}	0	0	0	0.001	0.004	0.014	0.064	0.114	0.210	0.346	0.448
0.0625 mmol l^{-1}	0	0	0	0.002	0.006	0.016	0.065	0.114	0.211	0.347	0.45
BHI control	0	-0.002	-0.002	0	0.003	0.015	0.0725	0.13	0.215	0.334	0.452

Appendix 2.2: Absorbance increases (405 nm) produced by various organisms in the presence of various naphthols.

	Time (mins)									
BHI 3,5-dihydroxynaphthalene-2-carbonyl-2,4,6-trimethylanilide										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	-0.003	-0.003	-0.003	-0.003	-0.003	-0.003	-0.003	-0.004	-0.004	-0.004
0.5 mmol l^{-1}	0	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0	0	0
0.25 mmol l^{-1}	0	0	-0.001	-0.001	0	-0.001	-0.001	-0.001	-0.001	-0.001	-0.001
0.125 mmol l^{-1}	0	-0.001	-0.001	-0.001	-0.001	-0.001	-0.001	-0.001	-0.001	-0.001	-0.001
0.0625 mmol l^{-1}	0	0	-0.001	-1E-03	-1E-03	-1E-03	-1E-03	-1E-03	-1E-03	-0.001	-0.001
BHI control	0	0	-0.002	-0.002	-0.001	-0.003	-0.002	-0.002	-0.001	-0.002	-0.002
S.aureus 3,5-dihydroxynaphthalene-2-carbonyl-2,4,6-trimethylanilide										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0	0	0	0.001	0.001	0.002	0.007	0.017	0.038	0.081
0.5 mmol l^{-1}	0	0.001	0.002	0.002	0.003	0.004	0.007	0.011	0.022	0.045	0.093
0.25 mmol l^{-1}	0	-0.001	-0.001	-1E-03	0	0.001	0.005	0.014	0.033	0.069	0.136
0.125 mmol l^{-1}	0	-0.001	0	0	0.001	0.002	0.008	0.018	0.045	0.09	0.174
0.0625 mmol l^{-1}	0	-0.001	-0.002	-1E-03	0	0.002	0.008	0.02	0.049	0.101	0.194
BHI control	0	-0.002	-0.002	-0.002	-1E-03	0.001	0.008	0.023	0.054	0.112	0.216
E.faecalis 3,5-dihydroxynaphthalene-2-carbonyl-2,4,6-trimethylanilide										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.002	0.001	0.002	0.004	0.006	0.009	0.014	0.019	0.028	0.052
0.5 mmol l^{-1}	0	0.001	0.002	0.002	0.005	0.008	0.017	0.033	0.063	0.099	0.187
0.25 mmol l^{-1}	0	0	-0.001	0	0.002	0.007	0.019	0.041	0.082	0.147	0.283
0.125 mmol l^{-1}	0	-0.001	-0.002	0	0.002	0.006	0.022	0.05	0.118	0.189	0.373
0.0625 mmol l^{-1}	0	-0.001	-0.002	-0.001	0.002	0.007	0.023	0.05	0.122	0.207	0.429
BHI control	0	-0.001	-0.002	-0.001	0.003	0.008	0.027	0.056	0.131	0.226	0.481
S.marcescens 3,5-dihydroxynaphthalene-2-carbonyl-2,4,6-trimethylanilide										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.001	0.001	0.001	0.002	0.002	0.005	0.021	0.092	0.293	0.47
0.5 mmol l^{-1}	0	0.001	0.002	0.003	0.003	0.005	0.009	0.022	0.059	0.174	0.252
0.25 mmol l^{-1}	0	-0.002	-0.001	0	0.002	0.004	0.011	0.028	0.072	0.161	0.264
0.125 mmol l^{-1}	0	0	0.001	0.002	0.003	0.006	0.014	0.034	0.085	0.209	0.329
0.0625 mmol l^{-1}	0	-0.001	0	0.001	0.002	0.006	0.015	0.037	0.099	0.227	0.348
BHI control	0	0	0	0.001	0.003	0.007	0.019	0.047	0.113	0.224	0.327
S.typhimurium 3,5-dihydroxynaphthalene-2-carbonyl-2,4,6-trimethylanilide										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	-0.001	-0.001	-0.001	0.004	0.012	0.033	0.089	0.204	0.329	0.388
0.5 mmol l^{-1}	0	0.001	0.001	0.001	0.006	0.014	0.036	0.084	0.164	0.269	0.36
0.25 mmol l^{-1}	0	-0.002	-0.003	-0.001	0.003	0.013	0.039	0.095	0.18	0.289	0.375
0.125 mmol l^{-1}	0	-0.002	-0.002	0	0.005	0.015	0.049	0.114	0.192	0.279	0.349
0.0625 mmol l^{-1}	0	-0.002	-0.002	0	0.006	0.018	0.053	0.125	0.218	0.3	0.389
BHI control	0	-0.001	-0.002	0	0.006	0.019	0.054	0.129	0.22	0.291	0.367
E.cloacae 3,5-dihydroxynaphthalene-2-carbonyl-2,4,6-trimethylanilide										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0	-0.001	0	0.005	0.009	0.028	0.079	0.18	0.24	0.336
0.5 mmol l^{-1}	0	0.001	0	0.001	0.007	0.012	0.034	0.092	0.191	0.263	0.387
0.25 mmol l^{-1}	0	-0.003	-0.004	-0.002	0.003	0.012	0.038	0.111	0.226	0.312	0.51
0.125 mmol l^{-1}	0	-0.003	-0.004	-0.002	0.005	0.012	0.048	0.127	0.244	0.36	0.56
0.0625 mmol l^{-1}	0	-0.002	-0.003	-0.002	0.004	0.013	0.049	0.147	0.246	0.362	0.572
BHI control	0	-0.002	-0.003	-0.001	0.004	0.015	0.053	0.155	0.248	0.38	0.572

Appendix 2.2: Absorbance increases (405 nm) produced by various organisms in the presence of various naphthols.

	Time (mins)									
K.pneumoniae 3,5-dihydroxynaphthalene-2-carbonyl-2,4,6-trimethylanilide										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	-0.003	-0.004	-0.004	0.001	0.005	0.019	0.052	0.112	0.222	0.413
0.5 mmol l^{-1}	0	-0.001	-0.002	-0.002	0.003	0.007	0.025	0.06	0.12	0.208	0.315
0.25 mmol l^{-1}	0	-0.006	-0.007	-0.006	-0.002	0.003	0.021	0.069	0.133	0.225	0.353
0.125 mmol l^{-1}	0	-0.004	-0.004	-0.003	0.002	0.008	0.051	0.111	0.231	0.391	0.482
0.0625 mmol l^{-1}	0	-0.006	-0.006	-0.005	0.001	0.007	0.035	0.098	0.241	0.446	0.608
BHI control	0	-0.007	-0.007	-0.005	-0.001	0.007	0.036	0.096	0.226	0.435	0.634
E.coli 3,5-dihydroxynaphthalene-2-carbonyl-2,4,6-trimethylanilide										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.001	0.002	0.002	0.003	0.008	0.014	0.031	0.068	0.11	0.163
0.5 mmol l^{-1}	0	0.001	0.003	0.002	0.003	0.008	0.019	0.042	0.097	0.139	0.236
0.25 mmol l^{-1}	0	0	0.002	0.002	0.004	0.011	0.027	0.067	0.128	0.205	0.33
0.125 mmol l^{-1}	0	-0.001	0.002	0.002	0.004	0.011	0.032	0.083	0.148	0.258	0.39
0.0625 mmol l^{-1}	0	-0.001	0.002	0.002	0.004	0.014	0.039	0.101	0.18	0.288	0.463
BHI control	0	-0.002	-0.001	0	0.002	0.013	0.045	0.123	0.202	0.314	0.454
BHI 1-naphthol										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.001	0.001	0.001	0.001	0.002	0.002	0.002	0.002	0.002	0.002
0.5 mmol l^{-1}	0	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.002
0.25 mmol l^{-1}	0	0	0.001	0.001	0.001	0.002	0.002	0.001	0.001	0.002	0.002
0.125 mmol l^{-1}	0	0.001	0.001	0.002	0.002	0.003	0.003	0.002	0.002	0.003	0.002
0.0625 mmol l^{-1}	0	0.001	-0.001	-0.001	0	-0.001	0	0	0.001	0.001	0.001
BHI control	0	0.001	0.002	0.003	0.001	0.003	0.003	0.001	0.002	0.002	0.001
S.aureus 1-naphthol										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.001	0.003	0.004	0.003	0.004	0.005	0.005	0.006	0.008	0.01
0.5 mmol l^{-1}	0	-0.001	-0.001	0	0.001	0.002	0.005	0.009	0.016	0.035	0.066
0.25 mmol l^{-1}	0	0.001	0.003	0.003	0.004	0.007	0.013	0.024	0.047	0.089	0.151
0.125 mmol l^{-1}	0	-0.003	-0.004	-0.003	-0.001	0.001	0.011	0.029	0.063	0.122	0.209
0.0625 mmol l^{-1}	0	0.001	0.001	0.001	0.003	0.007	0.019	0.047	0.096	0.17	0.28
BHI control	0	-0.003	-0.003	-0.003	-0.001	0.003	0.018	0.054	0.117	0.212	0.351
E.faecalis 1-naphthol										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.001	0.001	0.002	0.004	0.005	0.01	0.021	0.033	0.052	0.082
0.5 mmol l^{-1}	0	-0.001	-0.001	0	0.004	0.008	0.021	0.04	0.074	0.127	0.2
0.25 mmol l^{-1}	0	0.001	0.003	0.005	0.009	0.017	0.043	0.077	0.143	0.235	0.383
0.125 mmol l^{-1}	0	-0.002	-0.004	-0.002	0.003	0.012	0.044	0.094	0.177	0.308	0.486
0.0625 mmol l^{-1}	0	0.001	0.001	0.003	0.008	0.019	0.055	0.123	0.225	0.375	0.568
BHI control	0	0.001	0	0.001	0.008	0.022	0.055	0.139	0.268	0.425	0.655
S.marcescens 1-naphthol										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.002	0.003	0.003	0.003	0.005	0.004	0.004	0.004	0.004	0.004
0.5 mmol l^{-1}	0	-0.001	0	0	0.002	0.004	0.008	0.015	0.029	0.051	0.087
0.25 mmol l^{-1}	0	0.003	0.004	0.007	0.009	0.016	0.033	0.075	0.144	0.238	0.341
0.125 mmol l^{-1}	0	-0.002	-0.002	-0.001	0.002	0.01	0.029	0.08	0.163	0.27	0.364
0.0625 mmol l^{-1}	0	0.002	0.002	0.005	0.009	0.018	0.042	0.098	0.186	0.292	0.376
BHI control	0	-0.003	-0.001	0.001	0.003	0.013	0.035	0.081	0.159	0.253	0.334

Appendix 2.2: Absorbance increases (405 nm) produced by various organisms in the presence of various naphthols.											
		Time	(mins)								
S.typhimurium 1-naphthol											
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	-0.001	0	0.001	0.001	0.001	0.002	0.003	0.004	0.005	0.009
0.5 mmol l^{-1}	0	-0.001	0	0.001	0.005	0.011	0.032	0.078	0.139	0.196	0.267
0.25 mmol l^{-1}	0	0.002	0.003	0.005	0.01	0.023	0.058	0.129	0.207	0.289	0.327
0.125 mmol l^{-1}	0	-0.004	-0.005	-0.003	0.004	0.02	0.061	0.139	0.232	0.3	0.348
0.0625 mmol l^{-1}	0	0.001	0.002	0.004	0.012	0.03	0.073	0.151	0.251	0.309	0.369
BHI control	0	-0.003	-0.003	0	0.007	0.028	0.073	0.154	0.259	0.319	0.391
E.cloacae 1-naphthol											
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0	0	0	0.002	0	0.001	0.003	0.003	0.004	0.004
0.5 mmol l^{-1}	0	0	0	0	0.005	0.006	0.017	0.05	0.105	0.178	0.299
0.25 mmol l^{-1}	0	0	0.001	0.003	0.009	0.017	0.051	0.132	0.237	0.359	0.539
0.125 mmol l^{-1}	0	-0.002	-0.003	-0.001	0.007	0.017	0.072	0.17	0.272	0.441	0.577
0.0625 mmol l^{-1}	0	-0.001	0	0.002	0.01	0.024	0.082	0.188	0.287	0.471	0.593
BHI control	0	0.001	0	0.003	0.011	0.028	0.073	0.201	0.287	0.555	0.632
K.pneumoniae 1-naphthol											
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0	-0.001	-0.001	0	-0.001	0	0.001	0.002	0.001	0.002
0.5 mmol l^{-1}	0	-0.005	-0.006	-0.004	0	0.001	0.013	0.035	0.073	0.132	0.207
0.25 mmol l^{-1}	0	-0.002	-0.001	0.002	0.006	0.016	0.041	0.082	0.156	0.253	0.421
0.125 mmol l^{-1}	0	-0.006	-0.008	-0.006	0.002	0.013	0.052	0.116	0.23	0.384	0.634
0.0625 mmol l^{-1}	0	-0.002	-0.002	0.001	0.008	0.027	0.083	0.183	0.325	0.521	0.711
BHI control	0	-0.003	-0.004	-0.002	0.008	0.027	0.094	0.228	0.394	0.584	0.754
E.coli 1-naphthol											
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0	0.002	0.002	0	0.002	0.002	0.001	0.001	0.001	0.001
0.5 mmol l^{-1}	0	-0.002	0	-0.001	0	0.005	0.012	0.024	0.048	0.091	0.16
0.25 mmol l^{-1}	0	-0.003	0	0	0.002	0.011	0.029	0.07	0.142	0.258	0.414
0.125 mmol l^{-1}	0	-0.007	-0.005	-0.004	-0.001	0.01	0.038	0.104	0.21	0.347	0.501
0.0625 mmol l^{-1}	0	-0.003	-0.001	0	0.005	0.02	0.061	0.143	0.241	0.383	0.544
BHI control	0	-0.005	-0.005	-0.003	0.002	0.02	0.076	0.159	0.28	0.416	0.571

Appendix 2.2: Absorbance increases (405 nm) produced by various organisms in the presence of various naphthols.

	Time (mins)									
BHI anthranol										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0	0	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001
0.5 mmol l^{-1}	0	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
0.25 mmol l^{-1}	0	-0.001	-0.001	-0.002	-0.002	-0.001	-0.002	0	0	0	-0.001
0.125 mmol l^{-1}	0	-0.001	-0.001	-0.001	-0.001	-0.001	-0.001	0	-0.001	-0.001	-0.001
0.0625 mmol l^{-1}	0	0	0	-1E-03	-0.001	0	-0.001	0	0	0	0
BHI control	0	-0.002	-0.002	-0.002	-0.002	-0.002	-0.002	-0.002	-0.002	-0.002	-0.003
S.aureus anthranol										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.002	0.003	0.004	0.004	0.005	0.009	0.022	0.047	0.083	0.156
0.5 mmol l^{-1}	0	0.001	0.002	0.003	0.004	0.006	0.012	0.031	0.066	0.119	0.202
0.25 mmol l^{-1}	0	0.001	0.002	0.002	0.003	0.006	0.014	0.039	0.085	0.154	0.257
0.125 mmol l^{-1}	0	0.002	0.003	0.004	0.005	0.008	0.018	0.047	0.103	0.183	0.3
0.0625 mmol l^{-1}	0	0.001	0.002	0.002	0.004	0.008	0.018	0.049	0.104	0.184	0.304
BHI control	0	0	0	0	0.002	0.006	0.017	0.05	0.109	0.196	0.318
E.faecalis anthranol										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.002	0.004	0.006	0.009	0.015	0.03	0.083	0.156	0.26	0.492
0.5 mmol l^{-1}	0	0.002	0.004	0.005	0.009	0.017	0.048	0.115	0.216	0.342	0.516
0.25 mmol l^{-1}	0	0.001	0.002	0.004	0.007	0.016	0.051	0.13	0.258	0.426	0.613
0.125 mmol l^{-1}	0	0.001	0.002	0.004	0.008	0.02	0.056	0.149	0.295	0.488	0.668
0.0625 mmol l^{-1}	0	0	0.001	0.004	0.008	0.02	0.058	0.15	0.268	0.461	0.668
BHI control	0	-0.001	-0.001	0.001	0.004	0.013	0.048	0.146	0.295	0.491	0.683
S.marcescens anthranol										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.001	0.003	0.004	0.005	0.007	0.057	0.225	0.324	0.447	0.442
0.5 mmol l^{-1}	0	0.002	0.003	0.004	0.007	0.012	0.031	0.089	0.188	0.293	0.38
0.25 mmol l^{-1}	0	0.001	0.002	0.003	0.006	0.013	0.036	0.098	0.192	0.296	0.375
0.125 mmol l^{-1}	0	0.002	0.003	0.005	0.008	0.017	0.041	0.104	0.201	0.299	0.375
0.0625 mmol l^{-1}	0	0.001	0.001	0.003	0.006	0.015	0.04	0.1	0.196	0.292	0.317
BHI control	0	0.001	0.001	0.002	0.005	0.013	0.034	0.087	0.167	0.262	0.338
S.typhimurium anthranol										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.002	0.003	0.005	0.01	0.024	0.065	0.161	0.279	0.377	0.448
0.5 mmol l^{-1}	0	0.002	0.005	0.006	0.012	0.03	0.076	0.168	0.285	0.361	0.432
0.25 mmol l^{-1}	0	0.001	0.002	0.005	0.012	0.033	0.082	0.179	0.29	0.356	0.426
0.125 mmol l^{-1}	0	0	0.001	0.004	0.013	0.033	0.085	0.176	0.29	0.356	0.422
0.0625 mmol l^{-1}	0	-0.001	0	0.003	0.011	0.03	0.079	0.172	0.284	0.349	0.416
BHI control	0	0	0.001	0.004	0.01	0.027	0.07	0.149	0.257	0.314	0.373
E.cloacae anthranol										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.004	0.005	0.007	0.011	0.022	0.059	0.187	0.313	0.469	0.562
0.5 mmol l^{-1}	0	0.003	0.004	0.006	0.012	0.026	0.077	0.203	0.339	0.517	0.606
0.25 mmol l^{-1}	0	0.003	0.004	0.006	0.013	0.029	0.084	0.214	0.355	0.554	0.582
0.125 mmol l^{-1}	0	0.005	0.007	0.009	0.014	0.034	0.081	0.208	0.341	0.526	0.599
0.0625 mmol l^{-1}	0	0.002	0.003	0.006	0.013	0.029	0.086	0.197	0.34	0.533	0.583
BHI control	0	0.001	0.001	0.003	0.009	0.027	0.083	0.173	0.321	0.534	0.596

Appendix 2.2: Absorbance increases (405 nm) produced by various organisms in the presence of various naphthols.

	Time (mins)									
K.pneumoniae anthranol										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.003	0.005	0.008	0.012	0.022	0.074	0.173	0.34	0.503	0.767
0.5 mmol l^{-1}	0	0.004	0.005	0.008	0.015	0.033	0.09	0.222	0.4	0.605	0.778
0.25 mmol l^{-1}	0	0	0.002	0.005	0.013	0.032	0.092	0.215	0.378	0.598	0.76
0.125 mmol l^{-1}	0	0	0.002	0.005	0.013	0.032	0.096	0.231	0.41	0.645	0.794
0.0625 mmol l^{-1}	0	0.002	0.004	0.007	0.014	0.032	0.081	0.199	0.366	0.559	0.767
BHI control	0	-0.003	-0.002	0	0.008	0.028	0.083	0.213	0.406	0.587	0.751
E.coli anthranol										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.001	0.003	0.005	0.008	0.02	0.056	0.147	0.272	0.401	0.534
0.5 mmol l^{-1}	0	0.002	0.003	0.005	0.009	0.024	0.067	0.165	0.291	0.428	0.567
0.25 mmol l^{-1}	0	0	0.001	0.003	0.009	0.024	0.073	0.168	0.294	0.438	0.605
0.125 mmol l^{-1}	0	0	0.001	0.004	0.01	0.026	0.076	0.182	0.307	0.46	0.612
0.0625 mmol l^{-1}	0	0.001	0.002	0.004	0.01	0.025	0.072	0.176	0.305	0.443	0.589
BHI control	0	-0.004	-0.003	-0.001	0.005	0.02	0.068	0.154	0.283	0.438	0.599
BHI 4-chloro-1-naphthol										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	-0.001	-0.001	0	0	0	0	0.001	0.001	0.001	0.001
0.5 mmol l^{-1}	0	-1E-03	-1E-03	-1E-03	-0.001	-1E-03	-0.001	-0.001	-0.001	-0.001	0
0.25 mmol l^{-1}	0	-0.002	-0.002	-0.002	-0.002	-0.002	-0.002	-0.002	-0.002	-0.001	-0.002
0.125 mmol l^{-1}	0	-0.001	-0.001	-0.001	-0.001	0	0	0	0	0	0
0.0625 mmol l^{-1}	0	0	0	0.001	0	0.001	0.001	0.001	0.001	0.001	0.001
BHI control	0	-0.001	-0.001	-0.001	0	-0.001	-0.001	-0.001	-0.001	-0.001	0
S.aureus 4-chloro-1-naphthol										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0	0	0.001	0.001	0.001	0.002	0.002	0.003	0.003	0.003
0.5 mmol l^{-1}	0	0	0	0.001	0.001	0.002	0.002	0.002	0.002	0.002	0.002
0.25 mmol l^{-1}	0	0.001	0.001	0.002	0.002	0.002	0.003	0.003	0.003	0.003	0.003
0.125 mmol l^{-1}	0	-0.008	-0.008	-0.008	-0.008	-0.006	-0.005	-0.003	-0.002	0	0.002
0.0625 mmol l^{-1}	0	-0.001	-0.001	0	0.001	0.004	0.01	0.026	0.055	0.098	0.16
BHI control	0	-0.003	-0.004	-0.003	-0.001	0.003	0.015	0.05	0.116	0.207	0.331
E.faecalis 4-chloro-1-naphthol										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.001	0.001	0.001	0.002	0.002	0.002	0.003	0.003	0.003	0.003
0.5 mmol l^{-1}	0	0	0	0.001	0.001	0.001	0.001	0.002	0.002	0.002	0.002
0.25 mmol l^{-1}	0	0.001	0.001	0.002	0.002	0.003	0.004	0.006	0.007	0.008	0.011
0.125 mmol l^{-1}	0	-0.001	-0.001	0	0.002	0.007	0.014	0.035	0.062	0.103	0.162
0.0625 mmol l^{-1}	0	0	0.001	0.003	0.007	0.017	0.038	0.087	0.149	0.235	0.354
BHI control	0	-0.001	-0.001	0.002	0.006	0.022	0.054	0.134	0.289	0.475	0.685
S.marcescens 4-chloro-1-naphthol										
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0	0	0.001	0.002	0.001	0.001	0.002	0.002	0.002	0.003
0.5 mmol l^{-1}	0	0	0.001	0.001	0.001	0.001	0.002	0.002	0.002	0.002	0.003
0.25 mmol l^{-1}	0	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.002	0.002	0.002
0.125 mmol l^{-1}	0	-0.001	-0.001	0	0.001	0.003	0.006	0.016	0.034	0.065	0.108
0.0625 mmol l^{-1}	0	0.001	0.002	0.004	0.006	0.014	0.031	0.074	0.168	0.276	0.39
BHI control	0	-0.001	0	0.001	0.005	0.014	0.037	0.095	0.18	0.275	0.358

Appendix 2.2: Absorbance increases (405 nm) produced by various organisms in the presence of various naphthols.											
		Time	(mins)								
S.typhimurium 4-chloro-1-naphthol											
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.001	0.001	0.002	0.002	0.003	0.003	0.003	0.003	0.004	0.004
0.5 mmol l^{-1}	0	0.001	0.001	0.001	0.001	0.002	0.002	0.002	0.002	0.003	0.003
0.25 mmol l^{-1}	0	0.001	0.001	0.002	0.003	0.004	0.007	0.017	0.039	0.081	0.143
0.125 mmol l^{-1}	0	-0.003	-0.003	0	0.006	0.021	0.058	0.139	0.225	0.287	0.313
0.0625 mmol l^{-1}	0	0.003	0.004	0.007	0.013	0.031	0.074	0.157	0.255	0.303	0.343
BHI control	0	-0.003	-0.002	0.001	0.007	0.027	0.072	0.151	0.257	0.306	0.375
E.cloacae 4-chloro-1-naphthol											
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.002	0.003	0.003	0.003	0.004	0.004	0.004	0.005	0.005	0.005
0.5 mmol l^{-1}	0	0.003	0.003	0.003	0.003	0.003	0.004	0.004	0.004	0.004	0.004
0.25 mmol l^{-1}	0	0.002	0.003	0.003	0.004	0.005	0.009	0.017	0.035	0.074	0.154
0.125 mmol l^{-1}	0	0.003	0.004	0.006	0.011	0.022	0.055	0.153	0.237	0.396	0.533
0.0625 mmol l^{-1}	0	0.002	0.004	0.006	0.012	0.027	0.074	0.176	0.284	0.467	0.571
BHI control	0	0.002	0.003	0.006	0.012	0.032	0.093	0.204	0.318	0.535	0.605
K.pneumoniae 4-chloro-1-naphthol											
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0.002	0.002	0.002	0.003	0.003	0.003	0.004	0.004	0.004	0.004
0.5 mmol l^{-1}	0	0.002	0.003	0.002	0.003	0.003	0.003	0.004	0.004	0.004	0.004
0.25 mmol l^{-1}	0	0.001	0.002	0.002	0.003	0.006	0.012	0.025	0.044	0.078	0.136
0.125 mmol l^{-1}	0	-0.002	-0.002	0.001	0.006	0.017	0.046	0.115	0.219	0.343	0.511
0.0625 mmol l^{-1}	0	-0.004	-0.002	0.001	0.008	0.024	0.066	0.161	0.295	0.46	0.665
BHI control	0	0	0	0.004	0.009	0.03	0.081	0.198	0.37	0.578	0.754
E.coli 4-chloro-1-naphthol											
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
1 mmol l^{-1}	0	0	0	0	0	0.001	0.001	0.001	0.001	0.001	0.001
0.5 mmol l^{-1}	0	0	0	0	0.001	0.001	0.001	0.001	0.001	0.001	0.001
0.25 mmol l^{-1}	0	-0.001	-0.001	-0.001	-0.001	-0.001	-0.001	0	0	0	0
0.125 mmol l^{-1}	0	-0.005	-0.006	-0.006	-0.006	-0.005	-0.004	-0.002	-0.002	-0.002	-0.002
0.0625 mmol l^{-1}	0	-0.004	-0.004	-0.004	-0.002	0	0.006	0.019	0.044	0.093	0.173
BHI control	0	-0.004	-0.005	-0.004	0.001	0.015	0.058	0.147	0.251	0.408	0.551

Appendix 2.3: Growth of various organisms in the presence of a range of concentrations of L-alanyl-diethyl-*p*-phenylenediamine

	Time (mins)									
E.coli	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0	0	0.002	0.006	0.018	0.048	0.132	0.243	0.377	0.614
0.3125 mmol l^{-1}	0	-0	0	0.002	0.005	0.017	0.047	0.12	0.229	0.359	0.627
0.15625 mmol l^{-1}	0	0	0	0.002	0.005	0.017	0.046	0.119	0.23	0.352	0.648
0.07812 mmol l^{-1}	0	-0	-0	0	0.004	0.014	0.042	0.119	0.218	0.351	0.604
0.039 mmol l^{-1}	0	-0	0	0.002	0.005	0.015	0.045	0.13	0.23	0.364	0.633
BHI	0	0.001	0.002	0.003	0.007	0.02	0.053	0.136	0.253	0.389	0.668
K.pneumoniae	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0	-0	0.002	0.007	0.019	0.042	0.111	0.269	0.473	0.668
0.3125 mmol l^{-1}	0	-0	-0	0.001	0.007	0.027	0.067	0.152	0.319	0.526	0.83
0.15625 mmol l^{-1}	0	-0	-0	0	0.005	0.022	0.056	0.125	0.283	0.495	0.853
0.07812 mmol l^{-1}	0	-0.01	-0	-0	0.003	0.016	0.05	0.106	0.243	0.43	0.813
0.039 mmol l^{-1}	0	-0	-0	0	0.004	0.02	0.056	0.123	0.28	0.473	0.863
BHI	0	-0.01	-0	-0	0.003	0.019	0.053	0.11	0.273	0.495	0.843
E.cloacae	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0	-0	0	0.004	0.013	0.039	0.1	0.22	0.336	0.591
0.3125 mmol l^{-1}	0	-0	-0	1E-03	0.004	0.014	0.037	0.102	0.234	0.353	0.695
0.15625 mmol l^{-1}	0	-0	-0	-0	0.003	0.014	0.039	0.103	0.238	0.377	0.727
0.07812 mmol l^{-1}	0	-0	-0	0	0.003	0.015	0.042	0.117	0.243	0.379	0.688
0.039 mmol l^{-1}	0	-0	-0	-0	0.001	0.015	0.044	0.127	0.269	0.422	0.754
BHI	0	-0	-0	-0	0.003	0.017	0.05	0.142	0.286	0.454	0.764
S.typhimurium	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	0	1E-03	0.002	0.003	0.006	0.011	0.086	0.14	0.319	0.689
0.3125 mmol l^{-1}	0	-0	-0	-0	0.001	0.005	0.021	0.092	0.263	0.306	0.846
0.15625 mmol l^{-1}	0	-0	0	0	0.002	0.006	0.018	0.06	0.241	0.446	0.838
0.07812 mmol l^{-1}	0	-0	0	0.001	0.003	0.007	0.02	0.064	0.198	0.348	0.694
0.039 mmol l^{-1}	0	-0	0	0	0.002	0.007	0.019	0.042	0.205	0.309	0.71
BHI	0	-0	0	0.001	0.003	0.008	0.029	0.058	0.193	0.341	0.695
S.marcescens	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0	0	0.001	0.005	0.015	0.039	0.09	0.15	0.248	0.427
0.3125 mmol l^{-1}	0	-0	0	0.002	0.006	0.017	0.041	0.098	0.161	0.239	0.554
0.15625 mmol l^{-1}	0	-0	0	1E-03	0.005	0.015	0.038	0.097	0.159	0.288	0.448
0.07812 mmol l^{-1}	0	-0	-0	0.001	0.004	0.014	0.038	0.095	0.169	0.258	0.462
0.039 mmol l^{-1}	0	-0	-0	0	0.004	0.016	0.043	0.109	0.219	0.323	0.531
BHI	0	-0	-0	0.001	0.005	0.019	0.049	0.122	0.239	0.34	0.563
E.faecalis	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0	0	0.001	0.001	0.004	0.009	0.023	0.051	0.099	0.419
0.3125 mmol l^{-1}	0	0	0.003	0.003	0.004	0.007	0.012	0.025	0.049	0.103	0.457
0.15625 mmol l^{-1}	0	-0	0.002	0.002	0.003	0.006	0.012	0.024	0.05	0.109	0.475
0.07812 mmol l^{-1}	0	-0	0	0.001	0.002	0.005	0.012	0.029	0.059	0.116	0.538
0.039 mmol l^{-1}	0	-0	0.002	0.002	0.003	0.008	0.015	0.035	0.073	0.15	0.561
BHI	0	-0	0	0	0.003	0.007	0.015	0.036	0.084	0.17	0.571
S.aureus	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0	-0	0.001	0.002	0.003	0.008	0.012	0.023	0.039	0.275
0.3125 mmol l^{-1}	0	-0	0.001	0.001	0.001	0.003	0.006	0.015	0.031	0.051	0.287
0.15625 mmol l^{-1}	0	-0.01	-0	-0	-0.003	-0	0.007	0.015	0.029	0.062	0.317
0.07812 mmol l^{-1}	0	-0.01	-0	-0	-0.002	0	0.008	0.018	0.037	0.068	0.429
0.039 mmol l^{-1}	0	-0.01	-0.01	-0.01	-0.006	-0	0.003	0.014	0.035	0.071	0.512
BHI	0	-0.01	-0.01	-0.01	-0.008	-0.01	0	0.014	0.043	0.089	0.556
Control	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0.01	-0.01	-0.01	-0.005	-0	-0	-0	-0	-0.003	-0.004
0.3125 mmol l^{-1}	0	-0	-0.01	-0.01	-0.004	-0	-0	-0	-0.01	-0.004	-0.006
0.15625 mmol l^{-1}	0	-0	0	-0	0.001	0.003	0.005	0.004	-0	0.002	0.001
0.07812 mmol l^{-1}	0	-0	-0	-0	-0.003	-0	-0	-0	-0	-0.002	-0.003
0.039 mmol l^{-1}	0	0	-0	-0	-0.003	-0	-0	-0	-0	-0.003	-0.004
BHI	0	-0.01	-0	-0.01	-0.005	-0.01	-0.01	-0.01	-0.01	-0.005	-0.005

Appendix 2.3: Growth of various organisms in the presence of a range of concentrations of L-alanyl-4-aminophenol											
		Time	(mins)								
E.coli	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	0.001	0.002	0.004	0.01	0.021	0.055	0.143	0.268	0.422	0.672
0.3125 mmol l^{-1}	0	0	0.002	0.003	0.008	0.019	0.05	0.137	0.266	0.429	0.669
0.15625 mmol l^{-1}	0	0	0.002	0.003	0.008	0.019	0.051	0.135	0.258	0.405	0.664
0.07812 mmol l^{-1}	0	-0	0.001	0.002	0.007	0.019	0.047	0.133	0.23	0.359	0.63
0.039 mmol l^{-1}	0	-0	0	0.001	0.007	0.019	0.048	0.128	0.221	0.379	0.608
BHI	0	0.001	0.001	0.003	0.008	0.02	0.05	0.127	0.218	0.385	0.604
K.pneumoniae	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0.01	-0	-0	0.006	0.021	0.059	0.157	0.368	0.639	0.945
0.3125 mmol l^{-1}	0	-0	0	0.002	0.01	0.026	0.072	0.157	0.344	0.553	0.852
0.15625 mmol l^{-1}	0	-0	-0	0.001	0.009	0.023	0.06	0.145	0.33	0.54	0.848
0.07812 mmol l^{-1}	0	-0	0	0.002	0.009	0.024	0.068	0.148	0.328	0.54	0.797
0.039 mmol l^{-1}	0	-0	0	0.002	0.008	0.025	0.065	0.141	0.31	0.52	0.847
BHI	0	-0	-0	0.001	0.008	0.024	0.065	0.148	0.311	0.508	0.785
E.cloacae	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0	0	0	0.006	0.02	0.053	0.152	0.304	0.464	0.789
0.3125 mmol l^{-1}	0	-0	0	1E-03	0.007	0.019	0.052	0.151	0.303	0.472	0.772
0.15625 mmol l^{-1}	0	-0	-0	0	0.005	0.017	0.051	0.14	0.292	0.513	0.777
0.07812 mmol l^{-1}	0	-0	-0	0	0.005	0.018	0.055	0.165	0.25	0.496	0.771
0.039 mmol l^{-1}	0	-0	-0	-0	0.005	0.019	0.056	0.154	0.244	0.506	0.764
BHI	0	-0	-0	-0	0.004	0.019	0.056	0.151	0.24	0.489	0.738
S.typhimurium	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0	0	0.002	0.004	0.012	0.05	0.1	0.383	0.568	0.876
0.3125 mmol l^{-1}	0	0	0.001	0.001	0.003	0.007	0.021	0.056	0.169	0.314	0.555
0.15625 mmol l^{-1}	0	-0	0	0	0.002	0.005	0.015	0.036	0.133	0.269	0.5
0.07812 mmol l^{-1}	0	-0	0.001	0.002	0.004	0.008	0.021	0.052	0.132	0.25	0.213
0.039 mmol l^{-1}	0	-0	-0	0	0.002	0.007	0.018	0.048	0.124	0.229	0.439
BHI	0	-0	0	0.001	0.003	0.009	0.025	0.061	0.142	0.246	0.448
S.marcescens	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0	0	0.002	0.008	0.023	0.058	0.138	0.279	0.398	0.548
0.3125 mmol l^{-1}	0	-0	0	0.001	0.006	0.017	0.046	0.115	0.226	0.333	0.557
0.15625 mmol l^{-1}	0	-0	0.001	0.002	0.006	0.017	0.042	0.101	0.211	0.313	0.545
0.07812 mmol l^{-1}	0	-0	0.001	0.002	0.006	0.016	0.038	0.095	0.204	0.303	0.523
0.039 mmol l^{-1}	0	-0	0	0.001	0.005	0.015	0.039	0.098	0.199	0.298	0.52
BHI	0	-0	0	0.001	0.006	0.017	0.043	0.106	0.206	0.302	0.506
E.faecalis	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0.01	0	0.001	0.002	0.006	0.014	0.032	0.072	0.139	0.485
0.3125 mmol l^{-1}	0	-0	-0	0	0.002	0.005	0.014	0.033	0.073	0.149	0.548
0.15625 mmol l^{-1}	0	-0	0	0	0.002	0.007	0.017	0.036	0.081	0.15	0.559
0.07812 mmol l^{-1}	0	-0	0	0	0.002	0.006	0.015	0.035	0.074	0.15	0.52
0.039 mmol l^{-1}	0	-0	0.001	0.001	0.003	0.008	0.019	0.039	0.084	0.18	0.506
BHI	0	-0	0	0	0.002	0.007	0.018	0.04	0.092	0.189	0.495
S.aureus	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0	-0	0.001	0.001	0.004	0.009	0.021	0.034	0.065	0.401
0.3125 mmol l^{-1}	0	-0	0	0	0.002	0.005	0.011	0.022	0.035	0.062	0.446
0.15625 mmol l^{-1}	0	-0.01	-0.01	-0	-0.003	-0	0.005	0.016	0.035	0.069	0.479
0.07812 mmol l^{-1}	0	-0.01	-0	-0	-0.001	0.001	0.006	0.016	0.044	0.087	0.444
0.039 mmol l^{-1}	0	-0.01	-0	-0	-0.003	0	0.006	0.018	0.047	0.095	0.486
BHI	0	-0.01	-0.01	-0.01	-0.006	-0	0.005	0.019	0.052	0.109	0.51
Control	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0	-0	-0	-0.004	-0	-0	-0	-0	-0.004	0.006
0.3125 mmol l^{-1}	0	-0	-0	-0	-0.002	-0	-0	-0	-0	-0.003	-0.004
0.15625 mmol l^{-1}	0	-0	-0	-0	-0.003	-0	-0	-0	-0	-0.004	-0.004
0.07812 mmol l^{-1}	0	-0	-0	-0	-0.003	-0	-0	-0	-0	0	-0.001
0.039 mmol l^{-1}	0	-0.01	-0.01	-0	-0.004	-0.01	-0	-0	-0	-0.006	-0.002
BHI	0	-0.01	-0.01	-0.01	-0.008	-0.01	-0.01	-0.01	-0.01	-0.008	-0.009

Appendix 2.3: Growth of various organisms in the presence of a range of concentrations of L-alanyl-dimethyl-*p*-phenylenediamine											
		Time	(mins)								
E.coli	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	0	0.001	0.003	0.008	0.022	0.056	0.136	0.253	0.379	0.652
0.3125 mmol l^{-1}	0	0	0.001	0.003	0.008	0.022	0.058	0.143	0.259	0.393	0.634
0.15625 mmol l^{-1}	0	-0.001	0	0.002	0.007	0.022	0.06	0.146	0.26	0.414	0.618
0.07812 mmol l^{-1}	0	-0.002	-0.002	0.001	0.006	0.021	0.058	0.147	0.27	0.428	0.62
0.039 mmol l^{-1}	0	0.002	0.003	0.005	0.01	0.023	0.06	0.145	0.269	0.435	0.602
BHI	0	0	0	0.002	0.008	0.024	0.063	0.139	0.255	0.416	0.584
K.pneumoniae	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0.001	0	0.003	0.009	0.029	0.07	0.147	0.301	0.503	0.907
0.3125 mmol l^{-1}	0	-0.004	-0.004	-0.002	0.005	0.028	0.076	0.163	0.338	0.546	0.896
0.15625 mmol l^{-1}	0	0	0.001	0.004	0.012	0.032	0.077	0.171	0.344	0.543	0.897
0.07812 mmol l^{-1}	0	-0.005	-0.002	0.001	0.007	0.028	0.075	0.166	0.345	0.552	0.879
0.039 mmol l^{-1}	0	0	0.002	0.005	0.014	0.036	0.083	0.179	0.357	0.566	0.882
BHI	0	0.001	0.001	0.006	0.012	0.031	0.072	0.158	0.337	0.564	0.88
E.cloacae	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0.001	0	0.002	0.007	0.023	0.062	0.165	0.313	0.52	0.781
0.3125 mmol l^{-1}	0	-0.007	-0.008	-0.007	-0.002	0.015	0.057	0.165	0.316	0.526	0.776
0.15625 mmol l^{-1}	0	0	0.001	0.004	0.01	0.025	0.069	0.179	0.323	0.554	0.751
0.07812 mmol l^{-1}	0	-0.007	-0.006	-0.003	0.002	0.019	0.062	0.181	0.325	0.567	0.75
0.039 mmol l^{-1}	0	-0.003	-0.003	0	0.006	0.023	0.067	0.191	0.33	0.572	0.754
BHI	0	-0.006	-0.004	-0.001	0.005	0.023	0.068	0.183	0.345	0.572	0.76
S.typhimurium	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	0	0	0.001	0.004	0.01	0.025	0.067	0.179	0.307	0.538
0.3125 mmol l^{-1}	0	-0.003	-0.002	0	0.002	0.008	0.024	0.072	0.172	0.298	0.528
0.15625 mmol l^{-1}	0	0.003	0.004	0.005	0.008	0.015	0.029	0.079	0.187	0.309	0.592
0.07812 mmol l^{-1}	0	-0.004	-0.003	-0.001	0.001	0.007	0.024	0.075	0.183	0.297	0.599
0.039 mmol l^{-1}	0	0.002	0.004	0.005	0.008	0.014	0.03	0.08	0.2	0.378	0.689
BHI	0	-0.003	-0.004	-0.003	0	0.007	0.026	0.082	0.204	0.346	0.654
S.marcescens	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0.001	0	0.003	0.007	0.023	0.056	0.136	0.254	0.347	0.566
0.3125 mmol l^{-1}	0	-0.004	-0.002	0	0.006	0.021	0.057	0.14	0.256	0.351	0.566
0.15625 mmol l^{-1}	0	0.003	0.004	0.005	0.011	0.027	0.064	0.147	0.269	0.363	0.586
0.07812 mmol l^{-1}	0	-0.005	-0.004	-0.002	0.003	0.019	0.055	0.138	0.256	0.349	0.581
0.039 mmol l^{-1}	0	0.001	0.002	0.004	0.009	0.027	0.068	0.16	0.294	0.392	0.614
BHI	0	0.001	0.001	0.003	0.008	0.026	0.066	0.154	0.287	0.384	0.623
E.faecalis	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0.001	0.001	0.003	0.004	0.01	0.022	0.049	0.123	0.235	0.654
0.3125 mmol l^{-1}	0	-1E-03	0.001	0.002	0.004	0.011	0.027	0.056	0.133	0.249	0.646
0.15625 mmol l^{-1}	0	0.001	0.002	0.003	0.007	0.015	0.03	0.064	0.169	0.327	0.746
0.07812 mmol l^{-1}	0	-0.001	0.002	0.003	0.005	0.014	0.032	0.074	0.177	0.318	0.725
0.039 mmol l^{-1}	0	0	0.002	0.003	0.006	0.014	0.033	0.081	0.193	0.352	0.743
BHI	0	0.001	0.002	0.004	0.007	0.014	0.032	0.071	0.171	0.331	0.709
S.aureus	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0.002	0	0.001	0.001	0.004	0.012	0.031	0.067	0.123	0.684
0.3125 mmol l^{-1}	0	-0.006	-0.004	-0.003	-0.004	0	0.003	0.018	0.057	0.154	0.704
0.15625 mmol l^{-1}	0	0.001	0.001	0.003	0.004	0.01	0.02	0.039	0.1	0.188	0.755
0.07812 mmol l^{-1}	0	-0.005	-0.003	-0.003	-0.002	0.003	0.013	0.037	0.083	0.151	0.71
0.039 mmol l^{-1}	0	-0.005	-0.006	-0.006	-0.004	0.002	0.014	0.038	0.087	0.165	0.731
BHI	0	-0.003	0	0	0.002	0.008	0.02	0.043	0.098	0.19	0.771
Control	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0.002	-0.002	-0.002	-0.002	-0.001	-0.001	-0.001	-0.002	-0.001	0
0.3125 mmol l^{-1}	0	-0.003	-0.003	-0.003	-0.002	-0.002	-0.003	-0.002	-0.003	-0.001	-0.001
0.15625 mmol l^{-1}	0	-0.003	-0.004	-0.003	-0.003	-0.002	-0.003	-0.003	-0.002	-0.002	-0.003
0.07812 mmol l^{-1}	0	-0.004	-0.005	-0.005	-0.005	-0.004	-0.005	-0.004	-0.004	-0.003	-0.006
0.039 mmol l^{-1}	0	-0.001	-0.001	0.002	0	0	0	0	0	0	0
BHI	0	-0.001	-0.003	-0.002	-0.003	-0.002	-0.002	-0.002	-0.003	-0.002	-0.002

Appendix 2.3: Growth of various organisms in the presence of a range of concentrations of L-alanyl-4-amino-2,6-dibromophenol											
		Time	(mins)								
E.coli	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0.002	0	0.001	0.005	0.013	0.032	0.083	0.193	0.296	0.613
0.3125 mmol l^{-1}	0	-0.001	0	0.002	0.006	0.015	0.038	0.101	0.199	0.33	0.591
0.15625 mmol l^{-1}	0	-0.001	0	0.002	0.006	0.016	0.042	0.111	0.223	0.355	0.595
0.07812 mmol l^{-1}	0	-0.002	-0.001	0	0.005	0.016	0.042	0.116	0.22	0.38	0.582
0.039 mmol l^{-1}	0	0.002	0.003	0.004	0.009	0.02	0.051	0.123	0.218	0.37	0.559
BHI	0	-0.002	-0.002	-0.001	0.004	0.017	0.052	0.129	0.224	0.382	0.581
K.pneumoniae	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0.002	-0.001	0.002	0.009	0.026	0.067	0.15	0.312	0.517	0.687
0.3125 mmol l^{-1}	0	-0.005	-0.004	-0.001	0.006	0.026	0.07	0.16	0.342	0.555	0.803
0.15625 mmol l^{-1}	0	0	0.002	0.005	0.012	0.029	0.064	0.143	0.31	0.547	0.854
0.07812 mmol l^{-1}	0	-0.007	-0.006	-0.004	0.002	0.021	0.063	0.155	0.334	0.553	0.831
0.039 mmol l^{-1}	0	0	0.001	0.004	0.011	0.028	0.072	0.167	0.334	0.526	0.828
BHI	0	-0.002	-0.001	0.002	0.007	0.025	0.066	0.146	0.306	0.539	0.849
E.cloacae	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0.003	-0.001	0	0.005	0.018	0.047	0.127	0.233	0.423	0.719
0.3125 mmol l^{-1}	0	-0.007	-0.005	-0.004	0.001	0.015	0.049	0.147	0.26	0.491	0.711
0.15625 mmol l^{-1}	0	-0.001	0.001	0.003	0.009	0.022	0.058	0.159	0.276	0.516	0.739
0.07812 mmol l^{-1}	0	-0.008	-0.005	-0.003	0.002	0.017	0.055	0.165	0.247	0.499	0.717
0.039 mmol l^{-1}	0	-0.006	-0.005	-0.003	0.003	0.017	0.055	0.145	0.205	0.487	0.71
BHI	0	-0.006	-0.003	-0.001	0.004	0.02	0.062	0.165	0.258	0.505	0.725
S.typhimurium	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	0	0.001	0.002	0.004	0.008	0.018	0.046	0.113	0.215	0.457
0.3125 mmol l^{-1}	0	-0.003	-0.001	0	0.002	0.007	0.02	0.053	0.133	0.245	0.454
0.15625 mmol l^{-1}	0	0.002	0.003	0.004	0.007	0.012	0.024	0.058	0.14	0.248	0.439
0.07812 mmol l^{-1}	0	-0.003	-0.001	-0.002	0.001	0.007	0.022	0.059	0.136	0.236	0.443
0.039 mmol l^{-1}	0	0	0.001	0.003	0.005	0.011	0.025	0.064	0.149	0.255	0.464
BHI	0	0	-0.001	0.001	0.003	0.009	0.023	0.058	0.146	0.253	0.472
S.marcescens	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0.002	0	0.001	0.006	0.025	0.053	0.116	0.22	0.32	0.46
0.3125 mmol l^{-1}	0	-0.004	-0.001	0	0.004	0.016	0.045	0.111	0.219	0.325	0.479
0.15625 mmol l^{-1}	0	-0.001	0.001	0.003	0.007	0.02	0.049	0.119	0.228	0.333	0.505
0.07812 mmol l^{-1}	0	-0.008	-0.005	-0.004	0.001	0.013	0.043	0.11	0.215	0.312	0.49
0.039 mmol l^{-1}	0	0	0.004	0.005	0.01	0.023	0.05	0.114	0.214	0.307	0.489
BHI	0	-0.001	0	0.002	0.006	0.018	0.048	0.114	0.214	0.305	0.491
E.faecalis	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0.001	0.001	0.002	0.004	0.009	0.019	0.04	0.084	0.193	0.641
0.3125 mmol l^{-1}	0	-0.001	0.002	0.002	0.005	0.011	0.021	0.045	0.102	0.222	0.667
0.15625 mmol l^{-1}	0	0.001	0.003	0.004	0.007	0.014	0.028	0.06	0.132	0.293	0.73
0.07812 mmol l^{-1}	0	-1E-03	0.003	0.004	0.006	0.012	0.026	0.064	0.135	0.277	0.69
0.039 mmol l^{-1}	0	0.004	0.008	0.008	0.01	0.018	0.03	0.062	0.136	0.269	0.678
BHI	0	0.002	0.004	0.005	0.007	0.016	0.032	0.073	0.152	0.334	0.704
S.aureus	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0.002	0	0.001	0	0.002	0.003	0.003	0.004	0.005	0.005
0.3125 mmol l^{-1}	0	-0.006	-0.003	-0.004	-0.005	0	0.002	0.006	0.007	0.005	0.004
0.15625 mmol l^{-1}	0	0	0.001	0.002	0.003	0.005	0.009	0.019	0.026	0.033	0.09
0.07812 mmol l^{-1}	0	-0.004	-0.002	-0.001	-0.003	0.002	0.009	0.02	0.048	0.072	0.299
0.039 mmol l^{-1}	0	-0.004	-0.003	-0.003	-0.002	0.001	0.008	0.026	0.064	0.116	0.532
BHI	0	-0.005	-0.002	-0.001	-0.001	0.004	0.013	0.029	0.068	0.142	0.67
Control	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0.007	-0.007	-0.007	-0.006	-0.007	-0.007	-0.006	-0.007	-0.007	-0.007
0.3125 mmol l^{-1}	0	-0.009	-0.009	-0.009	-0.009	-0.009	-0.009	-0.009	-0.009	-0.01	-0.01
0.15625 mmol l^{-1}	0	-0.007	-0.007	-0.008	-0.007	-0.008	-0.008	-0.007	-0.007	-0.008	-0.008
0.07812 mmol l^{-1}	0	-0.009	-0.007	-0.008	-0.008	-0.007	-0.008	-0.008	-0.009	-0.009	-0.009
0.039 mmol l^{-1}	0	-0.009	-0.009	-0.009	-0.009	-0.01	-0.01	-0.01	-0.01	-0.01	-0.011
BHI	0	-0.008	-0.009	-0.008	-0.009	-0.009	-0.008	-0.009	-0.008	-0.009	-0.01

Appendix 2.3: Growth of various organisms in the presence of a range of concentrations of L-alanyl-4-amino-2,6-dichlorophenol											
		Time	(mins)								
E.coli	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0.001	0	0.002	0.007	0.02	0.054	0.148	0.228	0.369	0.644
0.3125 mmol l^{-1}	0	-0.001	0	0.002	0.007	0.019	0.055	0.147	0.251	0.412	0.593
0.15625 mmol l^{-1}	0	0.001	0.002	0.004	0.009	0.022	0.063	0.155	0.268	0.432	0.627
0.07812 mmol l^{-1}	0	-0.001	0	0.002	0.008	0.023	0.064	0.152	0.259	0.462	0.607
0.039 mmol l^{-1}	0	0	0	0.003	0.009	0.026	0.07	0.164	0.275	0.464	0.591
BHI	0	0.001	0.002	0.004	0.011	0.028	0.071	0.16	0.271	0.459	0.598
K.pneumoniae	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0.003	-0.001	0.003	0.012	0.032	0.086	0.195	0.382	0.615	0.813
0.3125 mmol l^{-1}	0	-0.002	-0.001	0.002	0.007	0.022	0.065	0.165	0.339	0.592	0.841
0.15625 mmol l^{-1}	0	-0.005	-0.003	0	0.005	0.018	0.051	0.133	0.283	0.536	0.813
0.07812 mmol l^{-1}	0	-0.004	-0.002	0.001	0.007	0.022	0.061	0.158	0.322	0.546	0.813
0.039 mmol l^{-1}	0	-0.005	-0.004	-0.002	0.005	0.023	0.069	0.167	0.339	0.548	0.813
BHI	0	-0.003	-0.001	0.001	0.008	0.026	0.073	0.173	0.332	0.535	0.821
E.cloacae	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0.003	-0.003	0.001	0.004	0.016	0.044	0.12	0.228	0.405	0.673
0.3125 mmol l^{-1}	0	-0.003	-0.002	0.001	0.006	0.019	0.058	0.171	0.291	0.484	0.718
0.15625 mmol l^{-1}	0	-0.004	-0.002	0	0.004	0.019	0.061	0.175	0.302	0.547	0.697
0.07812 mmol l^{-1}	0	-0.006	-0.004	-0.003	0.002	0.018	0.064	0.19	0.311	0.544	0.701
0.039 mmol l^{-1}	0	-0.006	-0.004	-0.002	0.004	0.022	0.067	0.196	0.32	0.536	0.71
BHI	0	-0.005	-0.003	-0.001	0.006	0.025	0.068	0.186	0.317	0.529	0.71
S.typhimurium	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	0	0	0.001	0.004	0.011	0.059	0.212	0.411	0.569	0.873
0.3125 mmol l^{-1}	0	0	0	0.001	0.003	0.007	0.016	0.091	0.137	0.355	0.74
0.15625 mmol l^{-1}	0	-0.003	-0.002	-0.001	0.002	0.007	0.023	0.061	0.175	0.34	0.59
0.07812 mmol l^{-1}	0	-0.001	0	0.001	0.005	0.011	0.027	0.068	0.157	0.298	0.477
0.039 mmol l^{-1}	0	-0.002	-0.002	-0.001	0.002	0.007	0.022	0.067	0.154	0.261	0.446
BHI	0	-0.002	-0.001	0.001	0.004	0.011	0.03	0.076	0.16	0.27	io
S.marcescens	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0.003	-0.002	0.001	0.007	0.019	0.052	0.107	0.181	0.291	0.591
0.3125 mmol l^{-1}	0	-0.003	-0.002	0	0.005	0.018	0.05	0.122	0.228	0.338	0.509
0.15625 mmol l^{-1}	0	-0.002	-0.001	0.001	0.006	0.02	0.054	0.134	0.236	0.339	0.516
0.07812 mmol l^{-1}	0	-0.004	-0.002	0	0.005	0.019	0.052	0.126	0.228	0.333	0.503
0.039 mmol l^{-1}	0	-0.004	-0.003	-0.001	0.004	0.018	0.051	0.123	0.223	0.312	0.483
BHI	0	-0.003	-1E-03	0	0.005	0.018	0.048	0.114	0.212	0.296	0.473
E.faecalis	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0.004	-0.004	-1E-03	0.001	0.005	0.015	0.036	0.078	0.177	0.498
0.3125 mmol l^{-1}	0	0	0.002	0.004	0.006	0.01	0.021	0.041	0.099	0.205	0.608
0.15625 mmol l^{-1}	0	-0.005	-0.001	-0.001	0.001	0.007	0.02	0.059	0.112	0.301	0.654
0.07812 mmol l^{-1}	0	-0.006	-0.002	-0.001	0.001	0.006	0.02	0.052	0.128	0.302	0.69
0.039 mmol l^{-1}	0	-0.006	-0.002	-0.001	0.001	0.008	0.024	0.07	0.158	0.332	0.714
BHI	0	-0.004	-0.002	-0.001	0.003	0.012	0.03	0.078	0.169	0.322	0.676
S.aureus	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0.003	-0.001	0	0.001	0.001	0.002	0.003	0.002	0.002	0
0.3125 mmol l^{-1}	0	-0.001	0	0	0	0.001	0.003	0.006	0.005	0.006	0.004
0.15625 mmol l^{-1}	0	-0.005	-0.002	-0.002	-0.002	-0.001	0.003	0.012	0.016	0.018	0.016
0.07812 mmol l^{-1}	0	-0.003	-0.001	-0.001	0	0.001	0.005	0.016	0.031	0.051	0.168
0.039 mmol l^{-1}	0	-0.006	-0.004	-0.004	-0.003	0	0.005	0.02	0.052	0.087	0.408
BHI	0	-0.007	-0.006	-0.006	-0.004	-0.001	0.008	0.029	0.077	0.164	0.691
Control	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
0.625 mmol l^{-1}	0	-0.002	-0.001	-0.001	-0.002	-0.002	-0.002	-0.002	-0.002	-0.002	-0.003
0.3125 mmol l^{-1}	0	-0.001	-0.001	-0.001	-0.001	-0.001	-0.001	-0.001	-0.001	-0.001	-0.001
0.15625 mmol l^{-1}	0	-0.002	-0.002	-0.002	-0.002	-0.002	-0.002	-0.002	-0.002	-0.002	-0.002
0.07812 mmol l^{-1}	0	-0.001	-0.001	0	0	0	0	0	0	0	0
0.039 mmol l^{-1}	0	-0.001	-0.001	0	-0.001	0	-0.001	0	-0.001	-0.001	-0.001
BHI	0	-0.003	-0.003	-0.003	-0.003	-0.002	-0.002	-0.002	-0.002	-0.002	-0.002

Appendix 2.4: Increases in absorbance* due to formation of blue indophenol generated by *E.coli* in the presence of varying concentrations of 1-naphthol and L-alanyl-diethyl-*p*-phenylenediamine.

*Absorbance was measured at 620 nm with 405nm readings subtracted to adjust for growth.

Section A: Total reaction volume : 50 µl, final E.coli concentration: 4 x 10^8 cfu/ml

	Time (min)								
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
2.5mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.006	0.01	0.01	0.013	0.017	0.02	0.02	0.027
2.5mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.006	0.01	0.02	0.028	0.037	0.045	0.05	0.058
2.5mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.009	0.02	0.03	0.039	0.052	0.065	0.07	0.084
2.5mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.008	0.02	0.03	0.046	0.059	0.071	0.09	0.096
2.5mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.005	0.02	0.03	0.043	0.059	0.075	0.09	0.092
2.5mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.005	0.02	0.03	0.046	0.067	0.081	0.08	0.09
2.5mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.004	0.02	0.03	0.049	0.06	0.062	0.07	0.067
2.5mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.001	0	0	0.004	0.003	0.004	0.01	0.006
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
1.25 mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.006	0.01	0.01	0.013	0.016	0.019	0.02	0.026
1.25 mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.006	0.01	0.02	0.028	0.038	0.045	0.06	0.06
1.25mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.009	0.02	0.03	0.04	0.049	0.059	0.07	0.072
1.25mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.006	0.02	0.03	0.041	0.051	0.061	0.07	0.077
1.25mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.004	0.01	0.03	0.041	0.06	0.078	0.09	0.104
1.25mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0	0.01	0.03	0.048	0.07	0.093	0.1	0.103
1.25mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.005	0.01	0.03	0.044	0.064	0.071	0.07	0.073
1.25mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	-0	0	0	0.003	0.003	0.003	0	0.005
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.625mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.006	0.01	0.01	0.013	0.016	0.018	0.02	0.025
0.625mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.007	0.01	0.02	0.029	0.037	0.042	0.05	0.051
0.625mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.007	0.02	0.02	0.032	0.04	0.046	0.05	0.057
0.625mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.003	0.01	0.02	0.033	0.043	0.05	0.06	0.061
0.625mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.004	0.01	0.02	0.032	0.046	0.06	0.07	0.085
0.625mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.004	0.01	0.02	0.031	0.042	0.053	0.06	0.074
0.625mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.006	0.01	0.02	0.032	0.042	0.049	0.06	0.066
0.625mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0	0	0	0.003	0.003	0.003	0	0.005
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.312mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.006	0.01	0.01	0.013	0.014	0.017	0.02	0.021
0.312mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.006	0.01	0.02	0.021	0.025	0.029	0.03	0.035
0.312mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.005	0.01	0.02	0.023	0.029	0.033	0.04	0.04
0.312mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.002	0.01	0.02	0.022	0.029	0.035	0.04	0.044
0.312mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.001	0	0.01	0.021	0.027	0.036	0.04	0.051
0.312mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.003	0.01	0.01	0.023	0.03	0.039	0.05	0.055
0.312mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	-0	0.01	0.01	0.021	0.029	0.037	0.04	0.05
0.312mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.005	0	0	0.002	0.004	0.005	0	0.006
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.16mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.004	0.01	0.01	0.011	0.011	0.012	0.01	0.014
0.16mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.005	0.01	0.01	0.015	0.017	0.019	0.02	0.024
0.16mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	7E-18	0.01	0.01	0.014	0.017	0.019	0.02	0.025
0.16mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	-0	0.01	0.01	0.016	0.02	0.023	0.03	0.029
0.16mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	1E-17	0	0.01	0.014	0.018	0.024	0.03	0.032
0.16mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.002	0.01	0.01	0.015	0.02	0.025	0.03	0.033
0.16mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.005	0.01	0.01	0.015	0.02	0.027	0.03	0.035
0.16mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.002	-0	0	0.002	0.004	0.004	0.01	0.006
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.022	0.02	0.02	0.023	0.023	0.024	0.02	0.024
0mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.004	0	0.01	0.007	0.006		0.01	0.007
0mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.004	0.01	0.01	0.008	0.009	0.008	0.01	0.008
0mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.002	0	0	0.006	0.006	0.007	0.01	0.007
0mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.004	0	0.01	0.004	0.006	0.006	0.01	0.006
0mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.003	0	0.01	0.002	0.004	0.004	0.01	0.006
0mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.002	0	0.01	0.003	0.006	0.004	0.01	0.005
0mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.005	0	0.01	0.004	0.007	0.006	0.01	0.007

Section B: Total reaction volume : 50 µl, final E.coli concentration: 8 x 10^8 cfu/ml									
Appendix 2.4 (continued):		**Time (min)**							
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
2.5mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.02	0.05	0.03	0.033	0.039	0.043	0.05	0.061
2.5mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.015	0.03	0.05	0.165	0.226	0.075	0.25	0.094
2.5mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.027	0.04	0.07	0.223	0.257	0.108	0.24	0.119
2.5mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.025	0.04	0.08	0.222	0.243	0.115	0.17	0.105
2.5mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.015	0.04	0.07	0.169	0.15	0.093	0.09	0.09
2.5mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.01	0.04	0.07	0.084	0.066	0.061	0.06	0.078
2.5mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.013	0.04	0.06	-0	-0.08	0.05	-0.3	0.057
2.5mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.003	0.03	0.01	-0.03	-0.04	0.025	-0.1	0.024
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
1.25 mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.015	0.04	0.02	0.008	0.016	0.042	0.06	0.054
1.25 mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.015	0.03	0.04	0.114	0.16	0.065	0.21	0.075
1.25mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.021	0.04	0.05	0.139	0.174	0.08	0.2	0.091
1.25mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.018	0.04	0.06	0.109	0.135	0.091	0.17	0.1
1.25mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.008	0.04	0.06	0.025	1E-03	0.113	-0	0.104
1.25mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.007	0.04	0.04	-0.02	-0.04	0.084	-0.1	0.071
1.25mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.005	0.03	0.03	0.013	0.007	0.049	-0.1	0.047
1.25mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	-0	0.03	0.01	0.011	0.008	0.019	-0	0.024
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.625mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.021	0.05	0.03	0.041	0.047	0.036	0.06	0.044
0.625mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.011	0.03	0.03	0.055	0.077	0.043	0.13	0.048
0.625mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.016	0.04	0.04	-0.17	-0.24	0.06	-0.3	0.069
0.625mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.009	0.03	0.04	-0.05	-0.09	0.066	-0.2	0.073
0.625mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.001	0.04	0.03	0.024	0.032	0.071	0.03	0.083
0.625mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.003	0.03	0.03	0.047	0.071	0.083	0.09	0.099
0.625mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.003	0.03	0.03	0.037	0.057	0.062	0.04	0.059
0.625mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0	0.03	0.01	-0.02	-0.02	0.015	-0	0.021
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.312mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.018	0.04	0.02	-0.12	-0.14	0.029	-0.1	0.03
0.312mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.009	0.03	0.02	-0.07	-0.1	0.03	-0.1	0.033
0.312mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.01	0.04	0.03	1E-03	-0.01	0.043	-0	0.05
0.312mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.005	0.03	0.03	0.041	0.049	0.049	0.06	0.058
0.312mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.002	0.03	0.02	0.048	0.058	0.049	0.07	0.059
0.312mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.002	0.04	0.02	0.031	0.046	0.051	0.07	0.063
0.312mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.002	0.03	0.02	-0.27	-0.33	0.046	-0.4	0.059
0.312mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0	0.04	0.01	-0.15	-0.19	0.013	-0.3	0.016
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.16mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.025	0.05	0.03	-0.04	-0.06	0.032	-0.1	0.033
0.16mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.006	0.03	0.02	-0.02	-0.02	0.022	-0	0.024
0.16mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.006	0.03	0.02	-0	0.001	0.029	0.01	0.034
0.16mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.001	0.04	0.01	-0.01	-0	0.032	0.02	0.04
0.16mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	1E-03	0.04	0.01	-0.09	-0.09	0.031	-0	0.042
0.16mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0	0.04	0.01	-0.05	-0.07	0.026	-0.1	0.034
0.16mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0	0.04	0.01	0.02	0.012	0.025	0.02	0.033
0.16mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.001	0.04	0.01	0.005	-0	0.011	-0	0.014
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.01	0.04	0.01	-0	-0	0.013	-0	0.014
0mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.005	0.04	0.01	-0.03	-0.02	0.01	-0	0.012
0mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.003	0.04	0.01	-0.23	-0.29	0.013	-0.3	0.013
0mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	-0	0.04	0	-0.12	-0.15	0.007	-0.2	0.007
0mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	-0	0.04	0	-0.07	-0.09	0.006	-0.1	0.007
0mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.002	0.04	0.01	-0.02	-0.02	0.008	-0	0.009
0mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.002	0.04	0.01	-0.02	-0.01	0.01	-0	0.012
0mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.002	0.04	0.01	-0.03	-0.03	0.009	-0	0.011

Section C: Total reaction volume : 50 µl, final E.coli concentration: 10^9 cfu/ml									
Appendix 2.4 (continued):	**Time (min)**								
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
2.5mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.035	0.07	0.05	0.053	0.151	0.101	0.45	0.121
2.5mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.022	0.05	0.08	0.297	0.396	0.125	0.57	0.152
2.5mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.034	0.06	0.11	0.424	0.517	0.192	0.66	0.233
2.5mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.022	0.06	0.11	0.393	0.479	0.193	0.55	0.225
2.5mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.008	0.06	0.1	0.301	0.334	0.174	0.39	0.178
2.5mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.005	0.05	0.08	0.211	0.235	0.128	0.28	0.127
2.5mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.006	0.05	0.06	0.011	0.01	0.094	0.02	0.092
2.5mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	-0	0.05	0	-0.05	-0.07	0.009	-0.1	0.015
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
1.25 mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.022	0.05	0.03	-0.02	-0.02	0.053	-0	0.068
1.25 mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.02	0.05	0.06	0.132	0.2	0.081	0.31	0.092
1.25mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.028	0.05	0.08	0.209	0.271	0.107	0.37	0.126
1.25mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.006	0.06	0.07	0.178	0.24	0.137	0.34	0.164
1.25mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	1E-03	0.05	0.05	0.186	0.251	0.128	0.35	0.159
1.25mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	-0	0.05	0.03	0.148	0.217	0.125	0.29	0.138
1.25mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	-0	0.05	0.02	0.105	0.118	0.072	0.13	0.074
1.25mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	-0	0.05	0	0.043	0.046	0.012	0.06	0.019
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.625mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.019	0.05	0.03	0.042	0.048	0.035	0.07	0.051
0.625mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.014	0.05	0.04	0.058	0.076	0.053	0.11	0.058
0.625mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.01	0.05	0.05	0.023	0.041	0.076	0.06	0.086
0.625mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.002	0.05	0.04	0.057	0.065	0.087	0.09	0.104
0.625mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	-0	0.05	0.04	0.085	0.108	0.094	0.15	0.116
0.625mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	-0	0.05	0.03	0.065	0.093	0.084	0.13	0.096
0.625mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	-0	0.05	0.03	0.059	0.088	0.078	0.11	0.081
0.625mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	-0	0.06	0	0.007	0.008	0.012	0.02	0.023
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.312mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.015	0.05	0.02	0.043	0.055	0.027	0.05	0.031
0.312mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.012	0.05	0.03	0.063	0.074	0.045	0.1	0.05
0.312mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.02	0.06	0.04	0.062	0.066	0.05	0.1	0.057
0.312mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.002	0.05	0.01	0.056	0.063	0.027	0.07	0.032
0.312mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.001	0.06	0.02	0.064	0.074	0.047	0.09	0.067
0.312mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.001	0.06	0.02	0.059	0.071	0.042	0.1	0.06
0.312mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	-0	0.05	0.01	-0.02	-0.03	0.036	-0.1	0.043
0.312mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	-0	0.06	0	-0.01	-0.02	0.011	-0	0.02
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.16mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.019	0.05	0.02	-0.02	-0.02	0.025	-0	0.029
0.16mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.01	0.05	0.02	-0.01	-0.01	0.035	0	0.041
0.16mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.004	0.05	0.02	-0	0.007	0.04	0.03	0.048
0.16mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.001	0.05	0.02	-0.02	0.003	0.042	0.02	0.053
0.16mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	-0	0.04	0.01	0.067	0.079	0.033	0.08	0.045
0.16mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0	0.05	0.01	0.074	0.08	0.035	0.11	0.047
0.16mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	-0	0.05	0.01	0.065	0.069	0.027	0.08	0.041
0.16mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	-0	0.05	0.01	0.046	0.048	0.011	0.05	0.018
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.011	0.05	0.02	0.055	0.061	0.009	0.04	-0.009
0mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.014	0.06	0.02	0.029	0.029	0.024	0.03	0.015
0mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.006	0.06	0.01	0.007	-0.01	0.021	-0.1	0.025
0mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	-0	0.05	0	0.006	0.006	0.006	-0	0.007
0mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.004	0.07	0.01	0.017	0.02	0.014	0.02	0.015
0mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.002	0.06	0.01	0.023	0.024	0.011	0.04	0.011
0mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	-0.01	0.06	-0	0.031	0.032	0.004	0.04	0.009
0mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.005	0.07	0.01	0.012	0.013	0.016	0.02	0.023

Section D: Total reaction volume : 50 µl, organism free control									
Appendix 2.4 (continued):	**Time (min)**								
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
2.5mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	1E-03	0	0	0.004	0.004	0.004	0.01	0.004
2.5mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0	0	0	0.002	0.001	0.002	0	0.001
2.5mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.001	0	0	0.002	1E-03	1E-03	0	0.001
2.5mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.001	0	0	0.002	0.001	0.001	0	0.001
2.5mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.003	0	0	0.003	0.002	0.002	0	0.002
2.5mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	1E-03	0	0	0.002	0.002	0.003	0	0.002
2.5mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0	0	0	0.001	0.001	0	0	0.001
2.5mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0	0	0	0	0	0	-0	-0.001
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
1.25 mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0	0	0	1E-03	1E-03	1E-03	0	0
1.25 mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0	0	0	0.001	0.002	0.002	0	0.001
1.25mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0	0	0	1E-03	0	0	0	0
1.25mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.002	0	0	0.002	0.002	0.002	0	0.002
1.25mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.001	0	0	0	0.001	0.001	0	0
1.25mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.001	0	0	0.002	0.001	0.001	0	0.002
1.25mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.001	0	0	0.001	0.001	0.001	0	0.002
1.25mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0	-0	0	0	0	0	0	0
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.625mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.001	0	0	0.002	0.001	0.001	0	0.002
0.625mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.001	0	0	0.002	0.002	0.002	0	0.002
0.625mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.002	0	0	0.002	0.002	0.002	0	0.002
0.625mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	-0	-0	-0	-0	-0.01	-0.01	-0	-0.004
0.625mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	-0	-0	-0	-0	0.001	-0	-0	-0.001
0.625mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.001	0	0	0.001	0.001	0.001	0	0.001
0.625mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	-0	-0	-0	-0	-0	-0	-0	-0.001
0.625mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.001	0	0	0.001	0.001	3E-18	0	0.001
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.312mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0	0	0	1E-03	1E-03	1E-03	0	0.002
0.312mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.001	0	0	0.002	0.002	0.001	0	0.002
0.312mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	-0	-0	-0	-0	-0	-0	-0	-0.002
0.312mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.002	0	0	0.002	0.002	0.002	0	0.002
0.312mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.019	0.02	0.02	0.019	0.019	0.018	0.02	0.018
0.312mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.012	0.01	0.01	0.012	0.013	0.012	0.01	0.012
0.312mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	-0	0	0	3E-18	-0	-0	-0	-1E-03
0.312mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0	0	0	0	0	0	0	0
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.16mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.002	0	0	0.002	0.002	0.002	0	0.001
0.16mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0	0	0	0	0	0	0	0
0.16mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.002	0	0	0.002	0.002	0.002	0	0.001
0.16mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.001	0	0	0.002	0.001	0.001	0	0.001
0.16mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.001	0	0	0.001	0.001	0.001	0	0
0.16mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	-0	0	0	0	0	-0	0	0
0.16mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	3E-18	-0	0	0	0	0	0	0
0.16mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0	0	0	1E-03	1E-03	0	0	0
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	1E-03	0	0	0.002	0.002	0.002	0	0.002
0mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.001	0	0	0.001	0.001	0.001	0	0.002
0mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	-0	0	0	-0	-0	-0	-0	-7E-18
0mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.001	0	0	0.001	0.001	0.001	0	0.001
0mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	3E-18	0	0	0	0.001	0	0	-0.001
0mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0	0	0	0.001	0	0	0	0
0mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	1E-03	-0	0	0	0	0	0	0
0mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0	0	0	1E-03	1E-03	0	0	0

Section E: Total reaction volume : 100 µl, organism-free control									
Appendix 2.4 (continued):	**Time (min)**								
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
2.5mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.005	0.01	0.01	0.009	0.01	0.011	0.01	0.012
2.5mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.001	0	0	0.003	0.002	0.003	0	0.003
2.5mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.001	0	0	0.002	0.003	0.002	0	0.002
2.5mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0	0	0	1E-03	1E-03	0.002	0	0.002
2.5mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.002	0	0	0.003	0.003	0.002	0	0.002
2.5mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0	0	0	0.002	0.002	0.002	0	0.002
2.5mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.001	0	0	0.001	0.003	0.002	0	0.002
2.5mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0	-0	0	0	0	-0	-0	-0.001
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
1.25 mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.002	0	0	0.005	0.005	0.005	0.01	0.005
1.25 mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.001	0	0	0.001	0.002	0.001	0	0.002
1.25mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	-0	-0	0	1E-03	1E-03	1E-03	0	0.001
1.25mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.001	0	0	0.002	0.002	0.002	0	0.001
1.25mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	1E-03	0	0	1E-03	1E-03	1E-03	0	0.001
1.25mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0	0	0	0.001	0.001	0.001	0	0.001
1.25mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0	0	0	0	0	0	0	0
1.25mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0	0	0	0	0.001	0.001	0	0.001
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.625mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.001	0	0	0.002	0.003	0.004	0	0.004
0.625mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.001	0	0	0.003	0.002	0.002	0	0.003
0.625mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.002	0	0	0.002	0.002	0.002	0	0.002
0.625mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.001	0	0	0.001	0.001	0.001	0	0.001
0.625mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.002	0	0	0.001	0.002	0.002	0	0.002
0.625mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0	-0	-0	-0	0	0	-0	0
0.625mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.001	0	0	0	0	0	0	0
0.625mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.001	0	0	0.001	0.001	0.001	0	0.001
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.312mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.002	0	0	0.003	0.004	0.004	0	0.004
0.312mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0	0	0	0	0	0.001	0	0.001
0.312mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.001	0	0	0.003	0.003	0.002	0	0.002
0.312mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	-0	-0	0	-0	0	0	0	0
0.312mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.001	0	0	0.001	0.001	0.002	0	0.001
0.312mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0	-0	-0	0	0	-0	-0	-0.001
0.312mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	-0	-0	-0	-0	-0	-0	-0	-0.001
0.312mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0	0	0	0.001	0	0.001	0	0.001
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.16mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.003	0	0	0.004	0.005	0.005	0.01	0.006
0.16mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0	0	0	1E-03	1E-03	1E-03	0	0.002
0.16mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.001	0	0	0.002	0.002	0.002	0	0.002
0.16mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0	0	0	0.001	0.001	0.002	0	0.002
0.16mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.001	0	0	0.002	0.001	0.002	0	0.002
0.16mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0	0	-0	0	0	-0	-0	-0.001
0.16mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0	0	-0	-0	-0	-0	-0	-0.001
0.16mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0	0	0	0	0	0	0	0
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.002	0	0	0.005	0.005	0.006	0.01	0.006
0mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	-0	0	0	1E-03	1E-03	0.002	0	0.003
0mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.004	0	0.01	0.005	0.005	0.005	0.01	0.005
0mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.001	0	0	0.001	0.001	0.002	0	0.001
0mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.002	0	0	0.002	0.003	0.003	0	0.003
0mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	-0	-0	-0	-0	-0	-0	-0	-0.002
0mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0	-0	-0	-0	-0	-0	-0	-1E-03
0mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0	0	0	0	0	0.001	-0	-0.001

Section F: Total reaction volume : 100 µl, final E.coli concentration: 4 x 10^8 cfu/ml									
Appendix 2.4 (continued):	Time (min)								
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
2.5mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.014	0.02	0.02	0.027	0.032	0.039	0.05	0.052
2.5mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.015	0.02	0.04	0.056	0.068	0.077	0.08	0.091
2.5mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.019	0.04	0.06	0.082	0.106	0.136	0.16	0.187
2.5mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.015	0.04	0.07	0.092	0.12	0.148	0.18	0.21
2.5mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.011	0.04	0.07	0.096	0.128	0.16	0.19	0.195
2.5mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.009	0.03	0.06	0.101	0.144	0.154	0.17	0.19
2.5mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.01	0.04	0.07	0.123	0.137	0.144	0.15	0.159
2.5mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.004	0	0	0.003	0.002	0.004	0.01	0.008
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
1.25 mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.013	0.02	0.02	0.026	0.032	0.039	0.04	0.051
1.25 mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.012	0.03	0.04	0.055	0.069	0.085	0.1	0.11
1.25mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.016	0.03	0.06	0.083	0.107	0.128	0.15	0.162
1.25mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.01	0.03	0.06	0.087	0.112	0.135	0.16	0.172
1.25mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.009	0.03	0.06	0.096	0.154	0.204	0.24	0.268
1.25mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.004	0.03	0.06	0.108	0.162	0.211	0.21	0.209
1.25mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.009	0.03	0.05	0.097	0.121	0.127	0.13	0.135
1.25mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.002	-0	0	0.003	0.003	0.003	0.01	0.008
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.625mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.013	0.02	0.02	0.023	0.029	0.036	0.04	0.047
0.625mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.011	0.03	0.04	0.052	0.067	0.078	0.09	0.092
0.625mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.015	0.03	0.05	0.073	0.088	0.102	0.11	0.12
0.625mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.008	0.03	0.05	0.076	0.089	0.113	0.13	0.135
0.625mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.009	0.02	0.05	0.076	0.123	0.135	0.17	0.188
0.625mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.006	0.02	0.04	0.07	0.094	0.118	0.15	0.173
0.625mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.004	0.02	0.04	0.06	0.08	0.1	0.12	0.153
0.625mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.002	0	0	0.005	0.008	0.005	0.01	0.008
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.312mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.012	0.01	0.02	0.019	0.021	0.026	0.03	0.034
0.312mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.011	0.02	0.03	0.042	0.05	0.057	0.06	0.066
0.312mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.013	0.03	0.04	0.051	0.061	0.069	0.08	0.08
0.312mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.001	0.02	0.04	0.054	0.077	0.082	0.09	0.101
0.312mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.005	0.02	0.03	0.049	0.064	0.08	0.1	0.11
0.312mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.004	0.01	0.03	0.048	0.061	0.077	0.08	0.107
0.312mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.005	0.02	0.03	0.048	0.06	0.075	0.09	0.102
0.312mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.001	0	0.01	0.007	0.009	0.007	0.01	0.009
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.16mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.011	0.01	0.02	0.017	0.02	0.022	0.02	0.026
0.16mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.011	0.02	0.03	0.033	0.037	0.042	0.05	0.048
0.16mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.008	0.02	0.03	0.038	0.043	0.048	0.05	0.057
0.16mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.005	0.01	0.02	0.034	0.04	0.048	0.06	0.069
0.16mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.003	0.01	0.02	0.031	0.041	0.051	0.06	0.066
0.16mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.004	0.01	0.02	0.03	0.04	0.05	0.06	0.065
0.16mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.005	0.01	0.02	0.03	0.037	0.047	0.06	0.064
0.16mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.002	0.01	0.01	0.006	0.008	0.009	0.01	0.009
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.009	0.03	0.02	0.011	0.016	0.014	0.01	0.015
0mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.006	0.01	0.01	0.009	0.009	0.009	0.01	0.009
0mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	-0.02	0.01	0.01	0.015	0.017	0.018	0.02	0.018
0mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.003	0.01	0.01	0.007	0.008	0.008	0.01	0.011
0mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.004	0	0.01	0.005	0.009	0.008	0.01	0.011
0mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.002	0.01	0	0.003	0.006	0.009	0.01	0.009
0mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.003	0.01	0.01	0.01	0.012	0.013	0.01	0.013
0mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.003	0.01	0.01	0.009	0.01	0.014	0.01	0.013
Section G: Total reaction volume : 100 µl, final E.coli concentration: 8 x 10^8 cfu/ml									

Appendix 2.4 (continued):	**Time (min)**								
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
2.5mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.102	0.1	0.06	0.067	0.079	0.096	0.12	0.133
2.5mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.086	0.09	0.1	0.138	0.161	0.173	0.19	0.226
2.5mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.079	0.08	0.14	0.2	0.257	0.306	0.35	0.399
2.5mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.087	0.09	0.18	0.248	0.315	0.385	0.41	0.446
2.5mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.083	0.08	0.16	0.243	0.296	0.319	0.35	0.368
2.5mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.08	0.08	0.17	0.238	0.26	0.268	0.27	0.263
2.5mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.081	0.08	0.15	0.17	0.178	0.18	0.18	0.176
2.5mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.081	0.08	0.01	0.011	0.013	0.021	0.03	0.041
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
1.25 mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.099	0.1	0.09	0.072	0.085	0.104	0.13	0.155
1.25 mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.085	0.09	0.1	0.13	0.148	0.168	0.18	0.196
1.25mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.078	0.08	0.12	0.152	0.179	0.206	0.23	0.252
1.25mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.088	0.09	0.14	0.186	0.22	0.253	0.28	0.303
1.25mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.085	0.09	0.12	0.182	0.246	0.316	0.37	0.392
1.25mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.089	0.09	0.09	0.133	0.168	0.213	0.28	0.326
1.25mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.083	0.08	0.07	0.099	0.116	0.15	0.2	0.206
1.25mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.089	0.09	0.01	0.014	0.009	0.014	0.02	0.031
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.625mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.088	0.09	0.06	0.059	0.067	0.076	0.09	0.094
0.625mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.08	0.08	0.08	0.09	0.101	0.109	0.11	0.118
0.625mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.089	0.09	0.1	0.114	0.126	0.138	0.15	0.157
0.625mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.091	0.09	0.08	0.12	0.147	0.167	0.19	0.198
0.625mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.089	0.09	0.06	0.095	0.134	0.17	0.2	0.221
0.625mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.092	0.09	0.07	0.099	0.139	0.177	0.21	0.236
0.625mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.091	0.09	0.05	0.083	0.123	0.159	0.16	0.168
0.625mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.092	0.09	0.01	0.008	0.009	0.008	0.01	0.022
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.312mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.092	0.09	0.05	0.044	0.053	0.061	0.06	0.065
0.312mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.085	0.09	0.06	0.069	0.076	0.084	0.09	0.092
0.312mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.088	0.09	0.06	0.075	0.087	0.096	0.11	0.11
0.312mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.092	0.09	0.06	0.081	0.093	0.111	0.13	0.136
0.312mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.092	0.09	0.04	0.065	0.088	0.108	0.13	0.143
0.312mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.088	0.09	0.03	0.053	0.074	0.099	0.12	0.143
0.312mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.091	0.09	0.04	0.051	0.069	0.089	0.11	0.138
0.312mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.093	0.09	0.01	0.009	0.01	0.01	0.01	0.014
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.16mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.089	0.09	0.04	0.042	0.047	0.05	0.05	0.057
0.16mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.074	0.07	0.04	0.043	0.049	0.055	0.06	0.062
0.16mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.1	0.1	0.05	0.064	0.073	0.08	0.09	0.093
0.16mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.088	0.09	0.03	0.046	0.059	0.07	0.08	0.087
0.16mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.096	0.1	0.02	0.034	0.046	0.059	0.07	0.08
0.16mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.092	0.09	0.02	0.033	0.043	0.053	0.07	0.123
0.16mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.098	0.1	0.03	0.036	0.046	0.053	0.06	0.072
0.16mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.094	0.09	0.01	0.013	0.011	0.012	0.01	0.016
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.083	0.08	0.03	0.025	0.027	0.028	0.03	0.032
0mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.085	0.09	0.02	0.021	0.023	0.025	0.03	0.03
0mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.086	0.09	0.02	0.026	0.027	0.029	0.03	0.033
0mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.097	0.1	0.01	0.013	0.014	0.016	0.02	0.019
0mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.095	0.1	0.01	0.011	0.011	0.013	0.01	0.014
0mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.097	0.1	0.01	0.01	0.01	0.012	0.01	0.014
0mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.095	0.1	0.01	0.009	0.01	0.009	0.01	0.015
0mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.096	0.1	0.01	0.007	0.012	0.013	0.02	0.018

Section H: Total reaction volume : 100 µl, final E.coli concentration: 10^9 cfu/ml									
Appendix 2.4 (continued):	Time (min)								
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
2.5mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.165	0.17	0.11	0.029	0.063	0.078	0.13	0.272
2.5mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.136	0.14	0.17	0.224	0.267	0.317	0.35	0.388
2.5mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.129	0.13	0.24	0.312	0.39	0.47	0.54	0.598
2.5mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.132	0.13	0.26	0.365	0.463	0.503	0.56	0.594
2.5mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.14	0.14	0.22	0.36	0.43	0.474	0.49	0.503
2.5mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.13	0.13	0.14	0.221	0.312	0.315	0.32	0.326
2.5mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.126	0.13	0.07	0.099	0.146	0.175	0.18	0.187
2.5mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.137	0.14	0.01	0.018	0.032	0.016	0.02	0.036
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
1.25 mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.154	0.15	0.06	0.033	0.023	0.031	0.06	0.131
1.25 mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.138	0.14	0.14	0.17	0.191	0.211	0.22	0.239
1.25mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.128	0.13	0.18	0.211	0.24	0.271	0.3	0.324
1.25mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.137	0.14	0.17	0.238	0.298	0.355	0.4	0.433
1.25mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.13	0.13	0.1	0.147	0.194	0.243	0.31	0.367
1.25mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.133	0.13	0.07	0.119	0.231	0.296	0.31	0.328
1.25mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.13	0.13	0.05	0.107	0.173	0.189	0.2	0.211
1.25mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.13	0.13	0.01	0.004	0.005	0.011	0.03	0.033
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.625mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.16	0.16	0.1	0.075	0.12	0.088	0.11	0.139
0.625mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.13	0.13	0.1	0.115	0.125	0.134	0.14	0.142
0.625mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.138	0.14	0.12	0.148	0.17	0.186	0.2	0.208
0.625mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.137	0.14	0.11	0.146	0.186	0.216	0.24	0.265
0.625mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.137	0.14	0.08	0.149	0.201	0.221	0.26	0.282
0.625mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.143	0.14	0.06	0.103	0.154	0.21	0.24	0.262
0.625mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.133	0.13	0.04	0.079	0.121	0.158	0.16	0.174
0.625mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.144	0.14	0.01	0.008	0.004	0.006	0.02	0.026
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.312mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.164	0.16	0.1	0.089	0.098	0.1	0.11	0.119
0.312mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.127	0.13	0.07	0.087	0.1	0.109	0.12	0.119
0.312mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.141	0.14	0.09	0.107	0.124	0.137	0.15	0.155
0.312mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.14	0.14	0.05	0.093	0.123	0.143	0.16	0.177
0.312mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.154	0.15	0.05	0.08	0.106	0.135	0.16	0.179
0.312mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.142	0.14	0.03	0.054	0.078	0.116	0.13	0.15
0.312mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.147	0.15	0.03	0.045	0.063	0.09	0.11	0.141
0.312mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.142	0.14	0.01	0.006	0.005	0.007	0.01	0.012
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.16mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.13	0.13	0.06	0.056	0.065	0.058	0.08	0.084
0.16mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.131	0.13	0.06	0.069	0.082	0.091	0.1	0.105
0.16mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.139	0.14	0.07	0.081	0.094	0.105	0.12	0.124
0.16mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.136	0.14	0.03	0.059	0.071	0.093	0.1	0.114
0.16mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.148	0.15	0.03	0.036	0.05	0.066	0.08	0.099
0.16mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.137	0.14	0.02	0.034	0.042	0.058	0.07	0.081
0.16mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.138	0.14	0.02	0.027	0.034	0.045	0.06	0.066
0.16mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.15	0.15	0.01	0.013	0.013	0.017	0.01	0.022
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.125	0.13	0.04	0.044	0.041	0.04	0.04	0.051
0mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.141	0.14	0.04	0.045	0.05	0.054	0.06	0.063
0mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.159	0.16	0.01	0.024	0.034	0.037	0.05	0.052
0mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.152	0.15	0.01	0.006	0.015	0.013	0.01	0.02
0mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.161	0.16	0.02	0.011	0.015	0.019	0.02	0.025
0mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.167	0.17	0.02	0.013	0.021	0.015	0.02	0.028
0mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.163	0.16	0.02	0.016	0.02	0.013	0.02	0.028
0mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.168	0.17	0.02	0.013	0.02	0.02	0.02	0.029

Section I: Total reaction volume : 200 µl, organism-free control									
Appendix 2.4 (continued):	**Time (min)**								
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
2.5mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.018	0.01	0.01	0.008	0.009	0.009	0.01	0.011
2.5mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.01	0.01	0.01	0.006	0.006	0.006	0.01	0.006
2.5mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.004	0	0	0.004	0.004	0.004	0	0.004
2.5mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.005	0	0	0.004	0.004	0.004	0	0.004
2.5mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.004	0	0.01	0.005	0.004	0.004	0	0.003
2.5mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.006	0	0.01	0.004	0.004	0.003	0	0.003
2.5mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.005	0.01	0.01	0.005	0.004	0.004	0	0.003
2.5mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.005	-0	-0	-0	-0	-0	-0	-0.002
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
1.25 mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.013	0	0	0.003	0.004	0.004	0	0.004
1.25 mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.01	0	0	0.002	0.003	0.003	0	0.004
1.25mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.005	0	0	0.004	0.003	0.004	0	0.003
1.25mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.005	0	0	0.002	0.003	0.002	0	0.002
1.25mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.003	0	0	0.002	0.002	0.002	0	0.002
1.25mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.006	0	0	0.002	0.002	0.002	0	0.002
1.25mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.003	0	0	1E-03	1E-03	1E-03	0	0.001
1.25mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.004	0	-0	0	-0	-0	0	-1E-03
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.625mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.014	0	0	0.004	0.004	0.004	0.01	0.004
0.625mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.01	0	0	0.002	0.002	0.002	0	0.002
0.625mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.009	0.01	0.01	0.006	0.006	0.006	0.01	0.006
0.625mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.007	0	0	0.003	0.003	0.003	0	0.002
0.625mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.006	0	0	0.003	0.003	0.003	0	0.004
0.625mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.006	0	0	1E-03	1E-03	1E-03	0	0
0.625mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.007	0	0	0.002	0.003	0.003	0	0.003
0.625mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.006	0	0	1E-03	0.002	0.001	0	0.001
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.312mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.012	0	0	0.002	0.002	0	0	0
0.312mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.01	0	0	0.002	0.002	0.002	0	0.003
0.312mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.005	0	0	0.004	0.004	0.004	0	0.004
0.312mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.006	0	0	0.001	0.001	0.001	0	0.002
0.312mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.007	0	0	0.003	0.003	0.003	0	0.003
0.312mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.008	0	0	0.003	0.002	0.002	0	0.003
0.312mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.003	0	0	0.001	0.002	0.002	0	0.001
0.312mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.007	0	0	1E-03	1E-03	0	0	0
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.16mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.014	0	0	0.003	0.004	0.004	0	0.005
0.16mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.01	0	0	0.002	1E-03	0.002	0	0.002
0.16mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.009	0.01	0	0.005	0.005	0.005	0	0.006
0.16mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.006	0	0	0.003	0.002	0.003	0	0.003
0.16mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.008	0	0	0.003	0.003	0.003	0	0.003
0.16mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.006	0	0	0	0.001	0	0	0
0.16mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.005	0	0	0.001	0.001	0.001	0	0.001
0.16mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.006	0	0	0	0.001	0.001	0	0
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.016	0	0	0.004	0.005	0.005	0	0.006
0mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.013	0	0	0.005	0.005	0.005	0	0.005
0mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.008	0.01	0.01	0.007	0.006	0.006	0.01	0.006
0mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.009	0	0	0.003	0.004	0.003	0	0.004
0mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.008	0	0	0.004	0.003	0.004	0	0.004
0mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.005	0	-0	-0	0	0	0	0
0mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.006	0	0	-0	0	-0	0	0
0mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.005	0	0	-0	-0	-0	-0	-0.001

Section J: Total reaction volume : 200 µl, final E.coli concentration: 4 x 10^8 cfu/ml									
Appendix 2.4 (continued):	**Time (min)**								
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
2.5mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.092	0.08	0.06	0.119	0.13	0.149	0.16	0.198
2.5mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.076	0.07	0.09	0.124	0.159	0.198	0.23	0.26
2.5mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.089	0.08	0.13	0.188	0.255	0.326	0.4	0.476
2.5mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.087	0.06	0.13	0.194	0.28	0.337	0.35	0.387
2.5mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.091	0.06	0.12	0.208	0.213	0.225	0.24	0.243
2.5mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.088	0.04	0.08	0.084	0.084	0.087	0.09	0.096
2.5mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.088	0.03	0.03	0.033	0.038	0.034	0.04	0.043
2.5mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.092	0.01	0.01	0.002	0	0.001	0.01	0.016
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
1.25 mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.088	0.05	0.07	0.105	0.122	0.154	0.17	0.191
1.25 mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.085	0.07	0.09	0.134	0.173	0.221	0.27	0.305
1.25mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.098	0.07	0.11	0.177	0.238	0.29	0.34	0.383
1.25mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.094	0.05	0.11	0.209	0.295	0.401	0.49	0.477
1.25mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.093	0.04	0.09	0.165	0.231	0.227	0.24	0.237
1.25mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.096	0.04	0.07	0.085	0.088	0.084	0.09	0.095
1.25mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.091	0.02	0.03	0.029	0.034	0.023	0.03	0.032
1.25mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.092	0.01	0.01	0	0.007	1E-03	0.01	0.013
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.625mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.088	0.05	0.07	0.098	0.125	0.148	0.17	0.179
0.625mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.087	0.07	0.09	0.126	0.153	0.178	0.2	0.219
0.625mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.097	0.06	0.11	0.158	0.212	0.245	0.28	0.296
0.625mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.097	0.04	0.08	0.136	0.187	0.229	0.28	0.31
0.625mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.095	0.03	0.06	0.106	0.144	0.172	0.21	0.232
0.625mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.095	0.03	0.05	0.07	0.091	0.086	0.09	0.096
0.625mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.087	0.02	0.03	0.03	0.03	0.03	0.03	0.031
0.625mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.098	0.01	0.01	0.013	0.012	0.011	0.01	0.005
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.312mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.089	0.05	0.07	0.084	0.097	0.101	0.11	0.119
0.312mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.1	0.06	0.08	0.099	0.114	0.129	0.14	0.153
0.312mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.102	0.04	0.07	0.117	0.135	0.162	0.19	0.208
0.312mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.098	0.03	0.05	0.088	0.122	0.156	0.18	0.201
0.312mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.1	0.03	0.05	0.074	0.105	0.128	0.16	0.179
0.312mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.097	0.02	0.04	0.055	0.072	0.08	0.08	0.094
0.312mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.097	0.02	0.03	0.036	0.036	0.034	0.03	0.053
0.312mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.098	0.01	0.01	0.014	0.013	0.013	0.01	0.024
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.16mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.085	0.04	0.05	0.064	0.073	0.079	0.08	0.088
0.16mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.101	0.05	0.06	0.071	0.08	0.089	0.1	0.105
0.16mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.098	0.03	0.05	0.071	0.094	0.107	0.13	0.139
0.16mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.1	0.02	0.04	0.058	0.076	0.095	0.12	0.13
0.16mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.097	0.02	0.02	0.046	0.06	0.077	0.09	0.11
0.16mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.095	0.02	0.03	0.039	0.047	0.06	0.07	0.079
0.16mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.094	0.01	0.02	0.028	0.033	0.034	0.04	0.041
0.16mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.101	0.01	0.01	0.013	0.014	0.014	0.01	0.016
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.091	0.03	0.03	0.028	0.029	0.031	0.03	0.034
0mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.077	0.01	0.02	0.024	0.023	0.024	0.03	0.027
0mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.068	0	0.01	0.009	0.011	0.01	0.01	0.015
0mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.067	0	0.01	0.007	0.007	0.011	0.01	0.01
0mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.084	0.02	0.02	0.023	0.024	0.025	0.03	0.027
0mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.077	0.01	0.01	0.009	0.01	0.01	0.01	0.013
0mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.095	0.01	0.02	0.017	0.019	0.019	0.02	0.022
0mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.108	0.01	0.02	0.018	0.021	0.023	0.03	0.025

Section K: Total reaction volume :200 µl, final E.coli concentration: 8 x 10^8 cfu/ml									
Appendix 2.4 (continued):	**Time (min)**								
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
2.5mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.065	0.15	0.24	0.361	0.469	0.556	0.66	0.704
2.5mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.102	0.21	0.31	0.475	0.631	0.81	7.82	7.684
2.5mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.082	0.26	0.45	0.679	0.888	0.99	8.19	8.136
2.5mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.035	0.2	0.43	0.584	0.642	0.679	0.7	0.706
2.5mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.022	0.12	0.28	0.289	0.304	0.312	0.32	0.311
2.5mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.004	0.05	0.1	0.095	0.108	0.12	0.13	0.137
2.5mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.006	0.03	0.04	0.033	0.045	0.061	0.07	0.081
2.5mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	-0.01	0	0.01	0.018	0.009	0.022	0.04	0.041
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
1.25 mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.075	0.15	0.23	0.307	0.373	0.428	0.5	0.541
1.25 mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.096	0.2	0.28	0.362	0.439	0.51	0.58	0.641
1.25mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.034	0.17	0.31	0.447	0.558	0.662	0.76	0.826
1.25mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.015	0.08	0.16	0.234	0.299	0.381	0.56	0.617
1.25mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.018	0.06	0.1	0.129	0.195	0.263	0.29	0.296
1.25mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.011	0.04	0.06	0.072	0.105	0.109	0.12	0.135
1.25mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.01	0.03	0.04	0.05	0.044	0.06	0.07	0.08
1.25mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.009	0.02	0.03	0.028	0.018	0.036	0.05	0.056
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.625mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.061	0.13	0.17	0.196	0.224	0.235	0.25	0.261
0.625mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.086	0.16	0.21	0.24	0.267	0.292	0.32	0.333
0.625mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.029	0.11	0.2	0.299	0.371	0.439	0.5	0.533
0.625mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.018	0.06	0.12	0.233	0.328	0.417	0.49	0.516
0.625mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.013	0.04	0.09	0.168	0.242	0.246	0.27	0.28
0.625mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0	0.02	0.06	0.097	0.094	0.107	0.12	0.129
0.625mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.011	0.03	0.04	0.048	0.046	0.057	0.07	0.074
0.625mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.013	0.02	0.02	0.026	0.026	0.027	0.04	0.047
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.312mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.055	0.1	0.13	0.151	0.166	0.173	0.18	0.185
0.312mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.063	0.11	0.15	0.171	0.19	0.208	0.22	0.232
0.312mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.022	0.06	0.12	0.187	0.233	0.276	0.31	0.336
0.312mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.005	0.04	0.09	0.135	0.186	0.239	0.29	0.324
0.312mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.011	0.04	0.06	0.095	0.131	0.176	0.22	0.23
0.312mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.012	0.03	0.05	0.068	0.095	0.102	0.11	0.116
0.312mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.007	0.02	0.03	0.041	0.044	0.045	0.05	0.057
0.312mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.009	0.02	0.02	0.025	0.029	0.027	0.03	0.038
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.16mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.043	0.08	0.1	0.12	0.135	0.143	0.15	0.158
0.16mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.059	0.09	0.11	0.132	0.149	0.163	0.18	0.186
0.16mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.015	0.04	0.07	0.107	0.138	0.166	0.19	0.208
0.16mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.011	0.03	0.05	0.076	0.098	0.124	0.15	0.174
0.16mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.012	0.03	0.05	0.058	0.077	0.093	0.11	0.137
0.16mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.002	0.02	0.03	0.043	0.052	0.065	0.08	0.102
0.16mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.021	0.04	0.04	0.052	0.056	0.063	0.07	0.076
0.16mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.018	0.03	0.03	0.036	0.041	0.041	0.04	0.043
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.043	0.05	0.06	0.066	0.07	0.074	0.08	0.079
0mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.012	0.02	0.03	0.027	0.03	0.032	0.03	0.031
0mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.003	0.01	0.01	0.018	0.018	0.016	0.02	0.015
0mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.012	0.02	0.03	0.03	0.033	0.034	0.04	0.038
0mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.012	0.02	0.02	0.027	0.03	0.032	0.03	0.034
0mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.003	0.01	0.02	0.021	0.021	0.022	0.03	0.026
0mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.002	0.01	0.02	0.021	0.021	0.022	0.02	0.025
0mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.004	0.01	0.02	0.024	0.026	0.032	0.03	0.035

Section L: Total reaction volume : 200 µl, final E.coli concentration: 10^9 cfu/ml									
Appendix 2.4 (continued):	**Time (min)**								
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
2.5mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.115	0.25	0.42	0.626	0.826	7.881	7.58	7.361
2.5mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.17	0.35	0.55	0.77	1.031	7.774	7.54	7.362
2.5mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.069	0.36	0.67	1	1.252	8.214	8.07	7.953
2.5mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.013	0.08	0.21	0.335	0.469	0.636	0.73	0.738
2.5mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.014	0.03	0.07	0.087	0.116	0.154	0.27	0.282
2.5mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.011	0.02	0.02	0.051	0.052	0.063	0.1	0.108
2.5mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.003	-0	0	0.025	0.031	0.041	0.04	0.053
2.5mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.008	-0	0.01	0.018	0.021	0.032	0.02	0.038
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
1.25 mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.11	0.24	0.33	0.401	0.477	0.572	0.65	0.734
1.25 mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.156	0.3	0.41	0.509	0.611	0.708	0.82	0.934
1.25mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.026	0.17	0.33	0.534	0.701	0.86	1.03	1.163
1.25mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	-0	0.05	0.09	0.237	0.467	0.556	0.6	0.627
1.25mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0	0.02	0.04	0.15	0.216	0.243	0.27	0.281
1.25mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	-0	0.01	0.02	0.082	0.077	0.095	0.11	0.126
1.25mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.004	0.01	0.01	0.031	0.022	0.038	0.05	0.068
1.25mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	-0	0	0	0.008	-0	-0	0.01	0.024
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.625mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.102	0.19	0.24	0.266	0.29	0.307	0.32	0.336
0.625mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.121	0.22	0.28	0.322	0.359	0.395	0.43	0.461
0.625mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.015	0.1	0.22	0.358	0.458	0.55	0.63	0.688
0.625mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.008	0.05	0.12	0.237	0.357	0.463	0.55	0.574
0.625mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.001	0.03	0.07	0.138	0.237	0.25	0.27	0.288
0.625mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.001	0.02	0.04	0.078	0.097	0.103	0.12	0.133
0.625mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	-0	0.01	0.02	0.027	0.028	0.032	0.04	0.053
0.625mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	-0	-0	0.01	0.009	0.006	0	0.01	0.02
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.312mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.076	0.14	0.18	0.208	0.228	0.243	0.25	0.26
0.312mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.073	0.15	0.19	0.23	0.26	0.283	0.3	0.321
0.312mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.017	0.07	0.14	0.216	0.279	0.335	0.38	0.418
0.312mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.008	0.04	0.07	0.12	0.178	0.238	0.29	0.336
0.312mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.001	0.02	0.04	0.065	0.095	0.143	0.2	0.236
0.312mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	-0	0.01	0.02	0.036	0.051	0.083	0.1	0.106
0.312mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.007	0.02	0.02	0.029	0.033	0.044	0.05	0.049
0.312mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.008	0.02	0.02	0.023	0.027	0.026	0.03	0.031
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.16mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.06	0.1	0.14	0.166	0.185	0.201	0.21	0.22
0.16mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.053	0.11	0.15	0.179	0.205	0.225	0.24	0.264
0.16mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.012	0.04	0.07	0.119	0.16	0.199	0.24	0.263
0.16mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.015	0.03	0.05	0.066	0.088	0.114	0.14	0.163
0.16mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.011	0.03	0.03	0.049	0.063	0.079	0.1	0.12
0.16mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.013	0.02	0.03	0.039	0.046	0.055	0.06	0.076
0.16mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.009	0.02	0.02	0.03	0.034	0.037	0.04	0.043
0.16mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.016	0.03	0.03	0.033	0.032	0.033	0.04	0.038
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0mmol^{-1} Ala-DEPPD/5mmol^{-1} 1-naphthol	0	0.059	0.08	0.09	0.094	0.099	0.106	0.11	0.116
0mmol^{-1} Ala-DEPPD/2.5mmol^{-1} 1-naphthol	0	0.045	0.09	0.1	0.108	0.115	0.121	0.12	0.129
0mmol^{-1} Ala-DEPPD/1.25mmol^{-1} 1-naphthol	0	0.016	0.02	0.02	0.021	0.019	0.029	0.04	0.039
0mmol^{-1} Ala-DEPPD/0.625mmol^{-1} 1-naphthol	0	0.019	0.03	0.03	0.033	0.036	0.041	0.05	0.046
0mmol^{-1} Ala-DEPPD/0.312mmol^{-1} 1-naphthol	0	0.009	0.02	0.02	0.022	0.027	0.03	0.04	0.04
0mmol^{-1} Ala-DEPPD/0.16mmol^{-1} 1-naphthol	0	0.013	0.02	0.02	0.029	0.034	0.037	0.04	0.049
0mmol^{-1} Ala-DEPPD/0.08mmol^{-1} 1-naphthol	0	0.018	0.03	0.03	0.033	0.037	0.042	0.04	0.052
0mmol^{-1} Ala-DEPPD/0mmol^{-1} 1-naphthol	0	0.015	0.02	0.03	0.03	0.034	0.039	0.05	0.045

Appendix 2.5: Increases in absorbance* due to formation of indophenol complex generated by a range of organisms in the presence of varying concentrations of 3,5-dihydroxy-2-naphthoic acid and L-alanyl-diethyl-*p*-phenylenediamine.

*Absorbance was measured at 620 nm with 405nm readings subtracted to adjust for growth.

	Time (mins)								
E.coli	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
2.5mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.12	0.15	0.19	0.23	0.3	0.36	0.46	0.573
2.5mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.12	0.19	0.3	0.39	0.49	0.54	0.65	0.652
2.5mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.14	0.25	0.4	0.58	0.88	1.11	1.24	1.4
2.5mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.07	0.25	0.42	0.62	0.91	0.98	1.1	1.152
2.5mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.16	0.39	0.66	0.77	0.82	0.83	0.851
2.5mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.1	0.33	0.54	0.57	0.57	0.56	0.547
2.5mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	-0	0.04	0.18	0.3	0.3	0.31	0.3	0.297
2.5mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.02	0.02	0.04	0.04	0.05	0.06	0.071
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
1.25 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.09	0.12	0.14	0.18	0.23	0.26	0.3	0.322
1.25 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.08	0.14	0.23	0.28	0.33	0.35	0.39	0.42
1.25 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.09	0.21	0.3	0.39	0.51	0.58	0.67	0.727
1.25 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.11	0.28	0.41	0.58	0.69	0.77	0.838
1.25 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.07	0.17	0.26	0.41	0.58	0.74	0.917
1.25 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.04	0.1	0.16	0.42	0.55	0.57	0.57
1.25 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.03	0.07	0.12	0.29	0.3	0.32	0.321
1.25 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0.03	0.03	0.02	0.05	0.07	0.076
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.625 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.09	0.11	0.13	0.15	0.17	0.18	0.19	0.204
0.625 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.1	0.16	0.21	0.24	0.26	0.26	0.28	0.296
0.625 mmol l^{-1} Ala-DEPPD/51.2mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.08	0.14	0.22	0.26	0.31	0.33	0.37	0.393
0.625 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.08	0.18	0.26	0.35	0.4	0.44	0.474
0.625 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.05	0.13	0.25	0.37	0.44	0.5	0.545
0.625 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.1	0.2	0.34	0.43	0.5	0.523
0.625 mmol l^{-1} Ala-DEPPD/0.0785mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.08	0.17	0.3	0.35	0.36	0.383
0.625 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0.02	0.03	0.03	0.03	0.04	0.056
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.313 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.1	0.12	0.13	0.14	0.16	0.16	0.18	0.182
0.313 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.08	0.13	0.16	0.19	0.21	0.21	0.22	0.229
0.313 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.12	0.16	0.19	0.22	0.24	0.25	0.266
0.313 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.05	0.12	0.18	0.23	0.26	0.29	0.307
0.313 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.02	0.07	0.14	0.22	0.26	0.29	0.328
0.313 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.03	0.06	0.11	0.19	0.25	0.29	0.33
0.313 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.02	0.05	0.08	0.15	0.21	0.24	0.285
0.313 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.01	0.01	0.02	0.02	0.03	0.03	0.037
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.156 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.08	0.09	0.1	0.11	0.12	0.12	0.13	0.132
0.156 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.11	0.14	0.16	0.18	0.2	0.21	0.22	0.228
0.156 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.06	0.09	0.14	0.16	0.19	0.2	0.22	0.228
0.156 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.04	0.08	0.12	0.17	0.19	0.2	0.219
0.156 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.03	0.05	0.08	0.13	0.17	0.19	0.213
0.156 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.04	0.05	0.08	0.12	0.15	0.18	0.205
0.156 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.04	0.06	0.1	0.12	0.14	0.17
0.156 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0	0.01	0.01	0.02	0.03	0.03	0.03	0.036
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.1	0.11	0.11	0.11	0.11	0.11	0.11	0.114
0 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.09	0.1	0.11	0.11	0.12	0.12	0.13	0.127
0 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.07	0.09	0.1	0.11	0.12	0.12	0.127
0 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.04	0.05	0.06	0.07	0.07	0.08	0.081
0 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0.03	0.04	0.04	0.05	0.05	0.055
0 mmol l^{-1} Ala-DEPPD/50.156mol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0.03	0.03	0.04	0.05	0.05	0.052
0 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.03	0.04	0.04	0.05	0.06	0.06	0.062
0 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.04	0.05	0.06	0.06	0.06	0.07

Appendix 2.5 (continued):									
		Time (mins)							
K.pneumoniae	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
2.5mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.02	0.04	0.05	0.09	0.11	0.13	0.125
2.5mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.09	0.14	0.17	0.21	0.26	0.3	0.376
2.5mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.1	0.19	0.29	0.34	0.36	0.38	0.445
2.5mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.17	0.3	0.42	0.48	0.56	0.6	0.538
2.5mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.15	0.26	0.57	0.64	0.63	0.63	0.613
2.5mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.12	0.16	0.38	0.45	0.47	0.47	0.417
2.5mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.1	0.25	0.39	0.37	0.36	0.28	0.371
2.5mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.09	0.09	0.25	0.21	0.09	0.1	0.094
	0	***30***	***60***	***90***	***120***	***150***	***180***	***210***	***240***
1.25 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	-0	0.01	0.01	0.02	0.02	0.03	0.034
1.25 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.07	0.12	0.16	0.17	0.18	0.18	0.201
1.25 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.13	0.24	0.33	0.45	0.5	0.44	0.429
1.25 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.11	0.24	0.36	0.46	0.52	0.52	0.508
1.25 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0	0.07	0.18	0.28	0.39	0.44	0.46	0.465
1.25 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0	0.05	0.14	0.26	0.34	0.34	0.4	0.336
1.25 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.05	0.14	0.22	0.26	0.2	0.15	0.25
1.25 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.07	0.07	0.07	0.08	0.07	0.08	0.087
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.625 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.01	0.01	0.01	0.02	0.02	0.03	0.036
0.625 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.07	0.12	0.15	0.17	0.17	0.17	0.16
0.625 mmol l^{-1} Ala-DEPPD/51.2mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.11	0.15	0.28	0.25	0.37	0.42	0.447
0.625 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.07	0.15	0.21	0.34	0.34	0.3	0.399
0.625 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0	0.02	0.12	0.18	0.21	0.25	0.28	0.273
0.625 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0	0.03	0.09	0.13	0.15	0.2	0.21	0.249
0.625 mmol l^{-1} Ala-DEPPD/0.0785mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0	0.03	0.09	0.12	0.18	0.2	0.23	0.242
0.625 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.06	0.06	0.06	0.06	0.06	0.07	0.08
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.313 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	-0	-0	0	-0	0.01	0.01	0.02	0.023
0.313 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.07	0.1	0.12	0.15	0.16	0.17	0.179
0.313 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.08	0.08	0.17	0.19	0.21	0.23	0.246
0.313 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.06	0.09	0.13	0.18	0.18	0.22	0.229
0.313 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.05	0.09	0.12	0.16	0.16	0.18	0.166
0.313 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	-0	0.02	0.05	0.08	0.11	0.1	0.12	0.131
0.313 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.08	0.1	0.12	0.13	0.14	0.154
0.313 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.09	0.09	0.1	0.09	0.1	0.1	0.107
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.156 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.01	0.01	0.02	0.03	0.02	0.03	0.034
0.156 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.06	0.08	0.09	0.11	0.11	0.13	0.136
0.156 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.06	0.07	0.09	0.11	0.13	0.14	0.16
0.156 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.04	0.06	0.07	0.1	0.12	0.14	0.157
0.156 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.03	0.04	0.06	0.08	0.09	0.1	0.117
0.156 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.02	0.03	0.05	0.06	0.07	0.08	0.092
0.156 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.05	0.06	0.07	0.08	0.09	0.102
0.156 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.06	0.09	0.09	0.09	0.09	0.09	0.098
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0	-0	0	0.01	0.01	0.01	0.02	0.022
0 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.06	0.07	0.07	0.08	0.08	0.08	0.08	0.084
0 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.06	0.06	0.06	0.07	0.08	0.09	0.09	0.095
0 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.02	0.03	0.02	0.04	0.05	0.066
0 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.06	0.06	0.04	0.06	0.04	0.05	0.08	0.092
0 mmol l^{-1} Ala-DEPPD/50.156mol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.04	0.05	0.06	0.05	0.06	0.066
0 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.06	0.07	0.07	0.08	0.08	0.09	0.094
0 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.05	0.07	0.07	0.08	0.09	0.09	0.091

Appendix 2.5 (continued):

	Time (mins)							
E.cloacae	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
2.5mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.05	0.1	0.16	0.7	1.32	1.891	2.147
2.5mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.14	0.2	0.26	0.49	0.55	0.223	0.566
2.5mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.09	0.25	0.4	0.54	0.91	1.2	7.536	8.051
2.5mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.06	0.19	0.4	1.09	1.34	1.31	0.84	1.48
2.5mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.08	0.13	0.2	1.19	1.28	1.14	0.43	1.112
2.5mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.08	0.1	0.75	0.79	0.81	0.321	0.854
2.5mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.05	0.1	0.38	0.43	0.49	0.376	0.651
2.5mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.1	0.04	0.05	0.14	0.418	0.346
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
1.25 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0	0.02	0	0.02	0.24	0.57	1.039	1.248
1.25 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.13	0.2	0.02	0.18	0.28	0.156	0.376
1.25 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.07	0.19	0.3	0.42	0.58	0.66	0.405	0.796
1.25 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.1	0.2	0.67	1	1.1	0.862	1.26
1.25 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.08	0.1	0.31	0.47	0.7	0.46	0.971
1.25 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.07	0.1	0.21	0.43	0.57	0.265	0.688
1.25 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.06	0.1	0.13	0.35	0.41	0.21	0.499
1.25 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.02	0	0.03	0.05	0.11	0.344	0.228
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.625 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	-0.01	-0	0	-0	0.05	0.13	0.359	0.254
0.625 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.07	0.1	-0	-0	0.01	-0.03	0.054
0.625 mmol l^{-1} Ala-DEPPD/51.2mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.12	0.2	0.13	0.25	0.33	0.243	0.462
0.625 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.07	0.1	0.33	0.48	0.62	0.633	0.757
0.625 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.05	0.1	0.29	0.46	0.64	0.625	0.749
0.625 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.05	0.1	0.25	0.45	0.59	0.682	0.734
0.625 mmol l^{-1} Ala-DEPPD/0.0785mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.05	0.1	0.2	0.37	0.47	0.46	0.498
0.625 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0	0.03	0.04	0.06	0.284	0.132
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.313 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.01	0	-0.1	-0	0.03	0.23	0.081
0.313 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.05	0.1	0.01	0.01	-0	0.026	-0.03
0.313 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.06	0.1	0.02	0.06	0.09	0.075	0.139
0.313 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.1	0.12	0.21	0.26	0.349	0.332
0.313 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.05	0.1	0.17	0.26	0.33	0.408	0.381
0.313 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.1	0.14	0.22	0.31	0.415	0.398
0.313 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.1	0.11	0.18	0.26	0.396	0.363
0.313 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.05	0.1	0.05	0.05	0.05	0.248	0.071
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.156 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.01	0	-0.1	-0.1	-0.03	0.182	0.013
0.156 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.06	0.07	0.1	0.02	0.04	0.05	0.189	0.067
0.156 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.06	0.1	-0	0.03	0.06	0.157	0.073
0.156 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.04	0.1	0.03	0.08	0.12	0.259	0.182
0.156 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0	0.05	0.09	0.13	0.273	0.184
0.156 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0	0.06	0.1	0.13	0.279	0.18
0.156 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.1	0.06	0.09	0.12	0.269	0.167
0.156 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.05	0.1	0.05	0.05	0.05	0.248	0.051
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0	-0.1	-0.1	-0.07	0.151	-0.05
0 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.04	0	-0.1	-0.1	-0.06	0.142	-0.06
0 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0	-0.1	-0.1	-0.05	0.137	-0.05
0 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.02	0	0	0.01	0.01	0.211	0.012
0 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.02	0	-0	0	0	0.209	0.012
0 mmol l^{-1} Ala-DEPPD/50.156mol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.03	0	0.02	0.03	0.03	0.231	0.039
0 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0	0.03	0.04	0.04	0.235	0.048
0 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0	0.03	0.04	0.04	0.231	0.04

Appendix 2.5 (continued):									
		Time (mins)							
S.typhimurium	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
2.5mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.09	0.1	0.15	0.23	0.33	0.608	0.592
2.5mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.07	0.09	0.1	0.64	0.95	1.03	1.011	1.085
2.5mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.06	0.12	0.2	0.82	0.98	0.99	0.642	0.914
2.5mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.07	0.2	0.74	1.04	1.18	0.777	1.064
2.5mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.05	0.1	0.71	1.01	1.13	0.811	0.994
2.5mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.03	0.1	0.45	0.62	0.63	0.576	0.628
2.5mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.04	0.1	0.4	0.42	0.46	0.498	0.475
2.5mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0	0.01	0	0.08	0.1	-0.05	0.164	-0.01
	0	***30***	***60***	***90***	***120***	***150***	***180***	***210***	***240***
1.25 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.04	0	0.08	0.07	0.09	0.21	0.103
1.25 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.06	0.13	0.2	0.58	0.79	0.8	0.651	0.692
1.25 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.09	0.2	0.6	0.81	0.88	0.395	0.564
1.25 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.06	0.2	0.45	0.63	0.72	0.54	0.731
1.25 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0	0.02	0.1	0.31	0.51	0.65	0.589	0.748
1.25 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.1	0.27	0.49	0.57	0.666	0.642
1.25 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.02	0	0.26	0.37	0.4	0.44	0.376
1.25 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.01	0	-0.1	-0	-0.05	0.136	-0.02
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.625 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.08	0.1	0.17	0.16	0.19	0.25	0.173
0.625 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.07	0.12	0.1	0.35	0.46	0.5	0.332	0.363
0.625 mmol l^{-1} Ala-DEPPD/51.2mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.07	0.1	0.39	0.49	0.56	0.298	0.504
0.625 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.03	0.1	0.23	0.43	0.45	0.359	0.491
0.625 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.03	0.1	0.2	0.29	0.38	0.392	0.44
0.625 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.01	0	0.13	0.24	0.31	0.339	0.371
0.625 mmol l^{-1} Ala-DEPPD/0.0785mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0	0.01	0	0.09	0.19	0.27	0.321	0.286
0.625 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0	0.02	0.02	0.03	0.193	0.031
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.313 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.09	0.1	0.14	0.14	0.14	0.239	0.135
0.313 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.06	0.11	0.1	0.17	0.22	0.23	0.182	0.204
0.313 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.06	0.08	0.1	0.25	0.27	0.31	0.212	0.29
0.313 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.04	0.1	0.17	0.24	0.28	0.256	0.276
0.313 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.1	0.14	0.21	0.24	0.253	0.232
0.313 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0	0.11	0.17	0.2	0.238	0.256
0.313 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.1	0.08	0.13	0.17	0.251	0.189
0.313 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.05	0	0.03	0.05	0.06	0.217	0.066
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.156 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.02	0	-0	-0	-0.01	0.15	-0.02
0.156 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.08	0.09	0.1	0.12	0.13	0.14	0.171	0.12
0.156 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.07	0.1	0.12	0.14	0.16	0.182	0.165
0.156 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.1	0.12	0.15	0.2	0.229	0.188
0.156 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.05	0.1	0.1	0.14	0.18	0.272	0.207
0.156 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.01	0	0.06	0.11	0.14	0.24	0.175
0.156 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0	0.03	0.07	0.11	0.231	0.131
0.156 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.03	0	-0	0.02	0.03	0.196	0.039
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	-0	0	-0.1	-0.1	-0.12	0.081	-0.14
0 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.04	0.1	0.05	0.05	0.04	0.172	0.027
0 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.06	0.06	0.1	0.1	0.1	0.08	0.21	0.072
0 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.06	0.1	0.08	0.1	0.09	0.24	0.068
0 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.05	0	0.06	0.08	0.08	0.228	0.04
0 mmol l^{-1} Ala-DEPPD/50.156mol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.03	0	0.03	0.03	0.04	0.207	0.042
0 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.04	0.1	0.03	0.03	0.04	0.21	0.044
0 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.04	0.1	0.03	0.03	0.05	0.216	0.057

Appendix 2.5 (continued):									
S.marcescens	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
2.5mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.06	0.04	0.07	0.08	0.1	0.09	0.09
2.5mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.1	0.15	0.19	0.21	0.23	0.24	0.26
2.5mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.08	0.19	0.3	0.39	0.49	0.57	0.63	0.73
2.5mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.22	0.4	0.63	0.91	1.14	1.3	1.65
2.5mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.11	0.25	0.41	0.57	0.76	0.91	1.03	1.19
2.5mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.13	0.27	0.37	0.49	0.58	0.64	0.7
2.5mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.11	0.23	0.31	0.37	0.38	0.37	0.37
2.5mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.04	0.03	0.04	0.05	0.05	0.05
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
1.25 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.05	0.05	0.06	0.07	0.06	0.07	0.07
1.25 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.11	0.17	0.23	0.27	0.3	0.3	0.32
1.25 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.09	0.21	0.31	0.42	0.54	0.64	0.7	0.75
1.25 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.16	0.3	0.46	0.6	0.71	0.81	0.9
1.25 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.12	0.23	0.34	0.43	0.5	0.54	0.61
1.25 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.11	0.19	0.28	0.35	0.39	0.42	0.48
1.25 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.09	0.16	0.21	0.26	0.3	0.32	0.34
1.25 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0.02	0.02	0.03	0.04	0.04	0.04
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.625 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.02	0.03	0.03	0.03	0.05	0.05
0.625 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.09	0.13	0.18	0.24	0.27	0.28	0.29
0.625 mmol l^{-1} Ala-DEPPD/51.2mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.06	0.15	0.25	0.34	0.47	0.56	0.61	0.66
0.625 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.1	0.2	0.32	0.45	0.56	0.66	0.74
0.625 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.07	0.15	0.21	0.33	0.43	0.5	0.57
0.625 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.06	0.12	0.18	0.26	0.34	0.4	0.46
0.625 mmol l^{-1} Ala-DEPPD/0.0785mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.04	0.09	0.13	0.21	0.25	0.3	0.34
0.625 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.01	0.01	0.02	0.03	0.03	0.03	0.04
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.313 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.03	0.03	0.05	0.04	0.06	0.05
0.313 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.08	0.1	0.13	0.17	0.2	0.22	0.24
0.313 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.09	0.16	0.21	0.29	0.35	0.39	0.43
0.313 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.07	0.13	0.2	0.28	0.35	0.4	0.45
0.313 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.04	0.09	0.15	0.23	0.29	0.33	0.37
0.313 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.04	0.07	0.13	0.19	0.23	0.26	0.28
0.313 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.06	0.1	0.15	0.17	0.19	0.21
0.313 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.01	0.03	0.03	0.04	0.05	0.05	0.05
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.156 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.04	0.02	0.04	0.04	0.06	0.07
0.156 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.06	0.07	0.08	0.09	0.12	0.13	0.15	0.16
0.156 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.08	0.11	0.15	0.18	0.21	0.23	0.25
0.156 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.05	0.08	0.12	0.17	0.2	0.23	0.26
0.156 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.06	0.1	0.14	0.17	0.2	0.22
0.156 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.04	0.06	0.09	0.13	0.16	0.18	0.19
0.156 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.05	0.08	0.11	0.13	0.15	0.16
0.156 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.02	0.03	0.04	0.05	0.05	0.06	0.06
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.04	0.04	0.02	0.02	0.04	0.05	0.07
0 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.06	0.07	0.08	0.07	0.08	0.08	0.08	0.09
0 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.05	0.06	0.06	0.06	0.06	0.06
0 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.04	0.05	0.06	0.06	0.06	0.06
0 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.04	0.05	0.06	0.06	0.06	0.06
0 mmol l^{-1} Ala-DEPPD/50.156mol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.04	0.05	0.06	0.06	0.06	0.06
0 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.02	0.03	0.04	0.05	0.05	0.05	0.05
0 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.04	0.05	0.06	0.06	0.07	0.07

Appendix 2.5 (continued):									
P.aeruginosa	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
2.5mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.05	0.06	0.06	0.06	0.07	0.07	0.08
2.5mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.15	0.17	0.17	0.17	0.17	0.18	0.18	0.18
2.5mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.19	0.26	0.28	0.28	0.28	0.28	0.29	0.28
2.5mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.12	0.24	0.29	0.31	0.33	0.35	0.37	0.38
2.5mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.11	0.22	0.27	0.33	0.4	0.45	0.46	0.48
2.5mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.07	0.14	0.22	0.29	0.31	0.36	0.43	0.48
2.5mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.07	0.14	0.21	0.22	0.28	0.33	0.35	0.37
2.5mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.04	0.04	0.04	0.06	0.04	0.03	0.02
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
1.25 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.03	0.03	0.04	0.04	0.04	0.05	0.05
1.25 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.14	0.16	0.17	0.17	0.17	0.17	0.18	0.18
1.25 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.18	0.25	0.28	0.29	0.29	0.3	0.3	0.3
1.25 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.08	0.19	0.25	0.29	0.32	0.35	0.38	0.41
1.25 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.06	0.13	0.2	0.27	0.34	0.37	0.38	0.35
1.25 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.08	0.15	0.23	0.29	0.31	0.29	0.33	0.38
1.25 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.06	0.12	0.19	0.21	0.2	0.23	0.28	0.34
1.25 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.04	0.04	0.04	0.04	0.05	0.05	0.04
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.625 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.05	0.06	0.06	0.07	0.07	0.07	0.07
0.625 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.14	0.17	0.18	0.18	0.19	0.19	0.2	0.2
0.625 mmol l^{-1} Ala-DEPPD/51.2mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.15	0.23	0.27	0.29	0.3	0.3	0.3	0.31
0.625 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.07	0.14	0.19	0.23	0.29	0.33	0.35	0.38
0.625 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.06	0.13	0.2	0.26	0.31	0.35	0.36	0.37
0.625 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.07	0.13	0.19	0.24	0.28	0.29	0.26	0.26
0.625 mmol l^{-1} Ala-DEPPD/0.0785mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.06	0.11	0.16	0.2	0.19	0.19	0.2	0.23
0.625 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.06	0.06	0.06	0.06	0.06	0.06	0.07	0.08
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.313 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.05	0.06	0.06	0.07	0.07	0.08	0.08
0.313 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.14	0.17	0.18	0.18	0.19	0.19	0.2	0.2
0.313 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.12	0.21	0.26	0.28	0.3	0.31	0.31	0.31
0.313 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.11	0.15	0.19	0.24	0.27	0.29	0.3
0.313 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.1	0.15	0.2	0.26	0.28	0.3	0.31
0.313 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.06	0.11	0.17	0.21	0.25	0.27	0.27	0.27
0.313 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.06	0.1	0.15	0.19	0.22	0.2	0.2	0.2
0.313 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.04	0.05	0.05	0.05	0.06	0.06	0.07
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.156 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.06	0.06	0.07	0.08	0.08	0.08	0.09
0.156 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.13	0.15	0.16	0.17	0.18	0.18	0.19	0.19
0.156 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.09	0.17	0.22	0.25	0.27	0.28	0.28	0.29
0.156 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.07	0.11	0.15	0.19	0.21	0.22	0.23
0.156 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.07	0.11	0.15	0.18	0.22	0.24	0.25	0.26
0.156 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.07	0.1	0.13	0.16	0.2	0.21	0.22	0.23
0.156 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.07	0.1	0.13	0.16	0.16	0.17	0.17
0.156 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.03	0.04	0.04	0.04	0.04	0.04	0.05
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.06	0.07	0.08	0.08	0.08	0.08	0.08
0 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.08	0.1	0.11	0.11	0.12	0.12	0.12	0.12
0 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.08	0.12	0.14	0.15	0.16	0.16	0.17	0.17
0 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.05	0.06	0.06	0.07	0.06	0.07	0.07
0 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.06	0.07	0.07	0.07	0.07	0.07	0.07
0 mmol l^{-1} Ala-DEPPD/50.156mol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.06	0.07	0.08	0.08	0.08	0.09	0.09	0.09
0 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.06	0.06	0.07	0.07	0.07	0.07	0.08
0 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.04	0.05	0.06	0.07	0.06	0.06

Appendix 2.5 (continued):									
		Time (mins)							
S.aureus	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
2.5mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.042	0.05	0.06	0.07	0.074	0.06
2.5mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.04	0.045	0.05	0.06	0.07	0.059	0.07
2.5mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.01	0.019	0.03	0.04	0.05	0.057	0.07
2.5mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.01	0.017	0.03	0.05	0.03	0.049	0.06
2.5mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.04	0.044	0.06	0.06	0.07	0.077	0.04
2.5mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.07	0.07	0.074	0.08	0.1	0.1	0.097	0.09
2.5mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.03	0.042	0.05	0.06	0.06	0.064	0.05
2.5mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0.037	0.04	0.05	0.06	0.06	0.04
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
1.25 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.01	0.007	0.02	0.02	0.03	0.046	0.02
1.25 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.044	0.04	0.05	0.04	0.031	0.07
1.25 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.01	0.023	0.03	0.04	0.04	0.026	0.02
1.25 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.01	0.016	0.02	0.03	0.02	0.031	0.03
1.25 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.035	0.04	0.05	0.05	0.057	0.06
1.25 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.01	0.02	0.02	0.03	0.03	0.032	0.03
1.25 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.01	0.02	0.02	0.03	0.02	0.025	0.03
1.25 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0.03	0.03	0.04	0.05	0.048	0.04
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.625 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0	0.01	-0	0	0.02	0.03	0.031	0.04
0.625 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.01	0.012	0.02	0.02	0.02	0.011	0.02
0.625 mmol l^{-1} Ala-DEPPD/51.2mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.033	0.04	0.04	0.03	0.05	0.04
0.625 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0.028	0.03	0.03	0.03	0.041	0.04
0.625 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.029	0.03	0.04	0.04	0.047	0.05
0.625 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0.026	0.03	0.04	0.04	0.045	0.05
0.625 mmol l^{-1} Ala-DEPPD/0.0785mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.031	0.04	0.04	0.05	0.048	0.04
0.625 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0.03	0.04	0.04	0.03	0.049	0.03
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.313 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.02	0.007	0.02	0.03	0.04	0.044	0.04
0.313 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.02	0.028	0.03	0.03	0.02	0.026	0.02
0.313 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.02	0.024	0.03	0.04	0.03	0.044	0.04
0.313 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.03	0.035	0.04	0.04	0.04	0.051	0.05
0.313 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.042	0.05	0.05	0.06	0.061	0.06
0.313 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.02	0.022	0.03	0.03	0.04	0.042	0.04
0.313 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0.029	0.03	0.04	0.04	0.047	0.05
0.313 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.04	0.05	0.05	0.06	0.061	0.06
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.156 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0.014	0.02	0.04	0.05	0.052	0.05
0.156 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0.033	0.04	0.04	0.01	-0	0.02
0.156 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.035	0.04	0.04	0.03	0.055	0.05
0.156 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.03	0.036	0.04	0.05	0.05	0.054	0.06
0.156 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.044	0.05	0.06	0.06	0.063	0.06
0.156 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.05	0.055	0.06	0.07	0.07	0.077	0.08
0.156 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.03	0.039	0.04	0.05	0.06	0.06	0.06
0.156 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.06	0.063	0.07	0.08	0.08	0.083	0.09
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.02	0.007	0.02	0.03	0.03	0.037	0.04
0 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0.028	0.02	0.03	0.03	-0.01	0.03
0 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.04	0.04	0.04	0.01	0.056	0.05
0 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.04	0.04	0.05	0.06	0.057	0.06
0 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.04	0.04	0.05	0.06	0.058	0.06
0 mmol l^{-1} Ala-DEPPD/50.156mol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.031	0.03	0.04	0.05	0.049	0.05
0 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.03	0.037	0.04	0.05	0.05	0.055	0.06
0 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.036	0.04	0.05	0.05	0.058	0.05

Appendix 2.5 (continued):									
		Time (mins)							
E.faecalis	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
2.5mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.048	0.05	0.06	0.06	0.068	0.08
2.5mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.01	0.016	0.02	0.04	0.05	0.056	0.06
2.5mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	-0.03	-0	-0.02	-0	0	0.02	0.029	0.04
2.5mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0	0.01	0.013	0.02	0.03	0.04	0.057	0.06
2.5mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	-0.01	-0	0.004	0.01	0.02	0.03	0.044	0.06
2.5mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.02	0.019	0.03	0.04	0.05	0.068	0.09
2.5mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	-0.01	-0	1E-03	0.01	0.02	0.03	0.049	0.07
2.5mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0	0	0.005	0.01	0.01	0.02	0.011	0.02
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
1.25 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.02	0.025	0.03	0.05	0.04	0.046	0.05
1.25 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.03	0.037	0.04	0.06	0.06	0.077	0.08
1.25 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0	0.01	0.014	0.02	0.03	0.04	0.042	0.04
1.25 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0.02	0.03	0.04	0.04	0.05	0.06
1.25 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0	0.01	0.013	0.02	0.03	0.04	0.047	0.06
1.25 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.02	0.019	0.02	0.04	0.04	0.052	0.07
1.25 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.02	0.018	0.02	0.03	0.04	0.052	0.07
1.25 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.01	0.01	0.017	0.02	0.03	0.03	0.033	0.04
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.625 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.046	0.06	0.07	0.08	0.082	0.08
0.625 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.06	0.06	0.066	0.07	0.08	0.09	0.089	0.1
0.625 mmol l^{-1} Ala-DEPPD/51.2mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.036	0.04	0.05	0.06	0.07	0.07
0.625 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.038	0.04	0.05	0.05	0.062	0.07
0.625 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0.025	0.03	0.04	0.05	0.043	0.06
0.625 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0.024	0.03	0.04	0.04	0.045	0.06
0.625 mmol l^{-1} Ala-DEPPD/0.0785mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.05	0.049	0.05	0.06	0.07	0.08	0.09
0.625 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.033	0.03	0.04	0.05	0.05	0.06
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.313 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.048	0.05	0.07	0.08	0.088	0.08
0.313 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.031	0.04	0.05	0.06	0.057	0.06
0.313 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.05	0.052	0.06	0.07	0.07	0.072	0.08
0.313 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.05	0.054	0.06	0.07	0.07	0.074	0.08
0.313 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.05	0.058	0.06	0.07	0.07	0.076	0.08
0.313 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.04	0.044	0.05	0.06	0.06	0.066	0.07
0.313 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.04	0.044	0.04	0.06	0.06	0.065	0.07
0.313 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.05	0.048	0.05	0.06	0.06	0.073	0.08
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0.156 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.05	0.05	0.06	0.07	0.08	0.084	0.08
0.156 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.04	0.043	0.05	0.06	0.07	0.069	0.07
0.156 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0.02	0.03	0.03	0.04	0.04	0.05
0.156 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.03	0.031	0.03	0.04	0.04	0.045	0.05
0.156 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.03	0.028	0.03	0.04	0.04	0.041	0.05
0.156 mmol l^{-1} Ala-DEPPD/0.156mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0.019	0.03	0.03	0.04	0.039	0.04
0.156 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0.028	0.03	0.04	0.04	0.042	0.05
0.156 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.03	0.032	0.03	0.04	0.04	0.042	0.05
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
0 mmol l^{-1} Ala-DEPPD/5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.05	0.052	0.06	0.07	0.07	0.077	0.08
0 mmol l^{-1} Ala-DEPPD/2.5mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.05	0.05	0.049	0.05	0.06	0.06	0.067	0.07
0 mmol l^{-1} Ala-DEPPD/1.25mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.03	0.029	0.04	0.04	0.04	0.044	0.05
0 mmol l^{-1} Ala-DEPPD/0.625mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.04	0.045	0.05	0.05	0.06	0.056	0.06
0 mmol l^{-1} Ala-DEPPD/0.313mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.04	0.04	0.047	0.05	0.06	0.06	0.055	0.06
0 mmol l^{-1} Ala-DEPPD/50.156mol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.03	0.032	0.04	0.04	0.04	0.043	0.05
0 mmol l^{-1} Ala-DEPPD/0.078mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.03	0.04	0.04	0.05	0.05	0.05	0.049	0.06
0 mmol l^{-1} Ala-DEPPD/0mmol l^{-1} 3,5-dihydroxy-2-naphthoic acid	0	0.02	0.02	0.03	0.03	0.04	0.04	0.042	0.05